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Tese de Doutoramento em Ciências
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Trabalho efectuado sob a orientação da
Professora Doutora Maria João Sousa
e da
Professora Doutora Maria Judite Almeida

Dezembro de 2008

À memória da minha mãe

A ti mãe, que espalhaste a alegria por onde passaste, que foste um exemplo para aqueles que pensam para lá dos seus horizontes. A ti mãe, pelo secreto Adeus!

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Baker's yeasts for use in frozen-dough technology: sugar utilization in freeze tolerant *Torulaspora delbrueckii* strains and elucidation of cryo-resistance mechanisms.

Abstract

Bread is a central dietary item in most countries of the world. Currently, frozen dough technology is extensively used in the baking industry to supply oven-fresh bakery products to consumers and to improve labor conditions for bakers. Since freeze–thaw stress affects the viability and activity of yeast cells, one serious disadvantage of this technology is a significant reduction in leavening activity during frozen storage. To develop improved baker's yeasts for use in frozen-doughs, yeast strains with high freeze tolerance as well as mechanisms of the freeze–thaw stress response in yeast cells, have been investigated with great interest.

Torulaspora delbrueckii strains PYCC 5321 and PYCC 5323, isolated from traditional corn and rye bread are of potential industrial interest since they display high resistance to osmotic and Na^+ injury and an exceptional freeze/ thaw tolerance, making them suitable for frozen dough technology. However, few reports exist on the genetics, biochemistry and physiology of *T. delbrueckii* in contrast to the vast knowledge on the traditional baker's yeast *Saccharomyces cerevisiae,* constituting a draw back for their commercial application.

Variability among *T. delbruecckii* strains PYCC 5321 and PYCC 5323 has been neither fully investigated nor reported by molecular typing. Therefore, we performed the molecular characterization of these yeast strains by both mitochondrial DNA restriction pattern analysis (RFLP's) and electrophoretic karyotyping, and showed that strain delimitation within the species *T. delbrueckii* by these methods is possible. In addition, we propose the use of RFLP's of mitochondrial DNA as an accessible molecular method to routinely discriminate *T. delbrueckii* strains.

For a better evaluation of the potential offered by this yeast to the baking industry we have also characterized sugar utilization patterns, and respiration/fermentation rates. Our results show that *T. delbruecki* behaves very similarly to *S. cerevisiae* with respect to sugar utilization and regulation patterns. However, when compared to a baker's yeast strain of *S. cerevisiae*, *T. delbrueckii* showed a higher contribution of respiration during aerobic fermentation of glucose, sucrose and maltose. This was evidenced by biomass yields determined in YP medium with either glucose, sucrose or maltose, which showed a very significant increase when high aeration rates were used (from 20% increase, in glucose or sucrose medium, to 80%, in maltose medium). This trait represents an advantage for the large-scale production of baker's yeast.

As shown for S. cerevisiae, we also have shown that sugar transport is the rate limiting step of sugar utilization in rich media in T. delbrueckii.

In Chapter 4 we have cloned and functionally characterized a new transporter gene from T. delbrueckii, **IGT1,** which encodes an intermediate-affinity glucose transporter. IGT1, is located upstream of LGT1, the first hexose transporter described in T. delbrueckii, and displays a high homology to this gene and to other yeast glucose transporter genes. Functional characterization of Igt1p in a S. cerevisiae hxt-null strain revealed that it encodes a transporter able to mediate the uptake of glucose, fructose and mannose. Furthermore, similarly to S. cerevisiae Hxt2p, apparent K_m of Igt1 transporter can be modulated by medium glucose concentration. Cells of S. cerevisiae hxt-null strain transformed with IGT1, when grown in 0.1% glucose displayed biphasic uptake kinetics with an intermediate- (K_m = 6.5 ± 2.0 mM) and a high-affinity (K_m = 0.10 ± 0.01 mM) component. Evidences that point to the existence of several hexose transporters with different glucose affinities and regulation in T. delbrueckii are also presented. Additionally, we have also established an improved gene disruption method for T. delbrueckii, and using this method constructed a Δlgt1 strain. Analysis of this mutant revealed that LGT1 disruption leads to a significant, although not severe, decrease in glucose transport in comparison with the wild-type strain.

Finally, special attention was given to yeast freeze resistance. The mechanisms of freeze tolerance and freeze sensitivity in yeast are still poorly understood and are an important issue to be solved for the development of bakers' yeast strains that are more suitable for the frozen-dough process. In a previous work it was shown that the higher freeze resistance of the T. delbrueckii strains under study, could be attributed to their higher capacity to preserve plasma membrane integrity. In S. cerevisiae a decrease in temperature induces the expression of many genes, some of which result in a cold-sensitivity phenotype when deleted. However, little is known about the role played by many cold-responsive genes, and the regulatory mechanisms that control their response. HSP12 gene is one of these genes. Furthermore, it was shown that Hsp12p could be localized at the plasma membrane making it a good candidate for a role in the preservation of membrane integrity during freezing. Chapter 6 focuses on the cold-shock responses of a Δhsp12 mutant, emphasizing the Hsp12p contribution to freeze tolerance and its relation with trehalose. We show that Hsp12p plays a role in cryoresistance, although the hsp12 null mutant revealed to be more resistant to freezing than the wild type strain. We found that stationary-phase cells of the Δhsp12 mutant have a higher intracellular trehalose concentration than wild type cells that could account for its higher resistance. However, heat-induced trehalose accumulation is impaired in this mutant. Overexpression of HSP12 in a Δtps1 strain (not able to accumulate trehalose) allowed to demonstrate a clear increase in resistance to freezing storage and also to heat stress.

Exploitation of yeast activities in the bread-making industry requires fundamental knowledge of their ecology, physiology, biochemistry and molecular biology. This knowledge, to which this work aimed to contribute, provides the base for genetic improvement strategies, and the new molecular methods for yeast identification and characterization, open up the possibility for future innovation in bakers' yeasts.

Leveduras de panificação para uso em massas congeladas: caracterização de estirpes criotolerantes de *Torulaspora delbrueckii* e mecanismos de resistência ao congelamento.

Resumo

O pão constitui um alimento essencial para uma dieta saudável a nível mundial. Actualmente, a utilização de massas congeladas na indústria da panificação apresenta várias vantagens, como o fornecimento aos consumidores de produtos de padaria e pastelaria sempre frescos e a melhoria das condições de trabalho, contribuindo para a sua expansão e aceitação. Os danos provocados pelo congelamento/ descongelamento afectam a viabilidade e a actividade das células de levedura, conduzindo a uma redução significativa na sua capacidade de levedação das massas.

Com o objectivo de desenvolver leveduras de panificação melhoradas para aplicação em massas panares congeladas, têm-se estudado e procurado estirpes de levedura com elevada crioresistência, assim como investigado mecanismos envolvidos na resposta ao stress provocado pelo congelamento/ descongelamento.

As estirpes de *Torulaspora delbrueckii* PYCC 5321 e PYCC 5323, isoladas do pão tradicional de milho e de centeio, possuem grande interesse com potencial aplicação na indústria da panificação. De facto, estas estirpes apresentam elevada resistência ao stresse osmótico e ao Na^+ e uma tolerância excepcional ao congelamento/ descongelamento, tornando-as apropriadas para o uso em massas panares congeladas. No entanto, existem poucos estudos de caracterização genética, bioquímica ou fisiológica da levedura *T. delbrueckii*, contrastando com o vasto conhecimento existente sobre a levedura tradicional de panificação *Saccharomyces cerevisiae*, o que constitui uma desvantagem para a aplicação comercial desta levedura não convencional.

As estirpes T. delbruecckii PYCC 5321 e PYCC 5323 foram anteriormente caracterizadas por estudos fisiológicos e bioquímicos, no entanto a sua variabilidade molecular não tinha ainda sido investigada. Por outro lado, também não se encontrava descrito um método expedito de tipagem molecular para diferenciação à estirpe de isolados de *T. delbruecckii*. Por essa razão, realizámos uma caracterização das estirpes PYCC 5321 e PYCC 5323, por análise de restrição de DNA mitocondrial (RFLP) e de cariotipagem electroforética, demonstrando que é possível a sua distinção/ diferenciação através destes dois métodos. Adicionalmente, propomos o uso de RFLP do DNA mitocondrial como um método molecular para a discriminação de rotina de estirpes de *T. delbrueckii*.

Para melhor avaliar o potencial biotecnológico desta levedura caracterizámos os seus padrões de utilização de açúcares e respectivas taxas de respiração/ fermentação. Os resultados mostraram que *T. delbruecki* se comporta de uma forma idêntica a *S. cerevisiae*, no que diz respeito aos padrões de utilização e regulação de açúcares. No entanto, quando comparada a uma estirpe de

panificação de *S. cerevisiae*, *T. delbrueckii* mostrou uma maior contribuição da respiração durante a fermentação aeróbia da glucose, sacarose e maltose. Este aspecto foi evidenciado pelo aumento significativo dos rendimentos em biomassa, determinados em meio YP suplementado com os diferentes açúcares e usando taxas de arejamento elevadas. Esta característica representa uma clara vantagem para a produção em larga escala de levedura de panificação.

De acordo com o descrito em diferentes trabalhos para *S. cerevisiae*, os estudos aqui descritos mostraram que em *T. delbrueckii* o transporte constitui o passo limitante no consumo de maltose e glucose. No Capítulo 4 descreve-se a clonagem e caracterização funcional de um novo gene transportador de *T. delbrueckii*, *IGT1*, que codifica um transportador com afinidade intermédia para a glucose. Este gene, localiza-se a montante e na mesma cadeia do gene *LGT1*, o primeiro transportador de hexoses descrito em *T. delbrueckii*. Ambos os genes possuem elevada homologia com outros genes transportadores de glucose em leveduras. A caracterização funcional da proteína Igt1p na estirpe mutante *hxt* de *S. cerevisiae* revelou que este gene codifica um transportador capaz de mediar o transporte de glucose, frutose e manose. Tal como se verificou para o transportador Hxt2p de *S. cerevisiae*, o K_m do transportador Igt1 pode ser modulado pela concentração de glucose no meio de cultura. Células da estirpe mutante de *S. cerevisiae*, transformadas com o gene *IGT1* e cultivadas em glucose 0,1%, mostraram uma cinética de transporte de glucose bifásica, constituída por uma componente de afinidade intermédia (K_m = 6.5 ± 2.0 mM) e outra de alta afinidade (K_m= 0.10 ± 0.01 mM). Os resultados apresentados sugerem ainda a existência de outros transportadores de hexoses com diferentes afinidades para a glucose em *T. delbrueckii*. Adicionalmente, foi desenvolvido um método melhorado de interrupção de genes em *T. delbrueckii*, com o qual foi possível obter a estirpe mutante Δ*lgt1*. A interrupção do gene *LGT1* resultou num decréscimo significativo embora não acentuado do transporte de glucose comparativamente com a estirpe selvagem.

Por fim foi dada especial atenção à resistência ao congelamento em leveduras. Os mecanismos de tolerância e sensibilidade ao congelamento em leveduras constituem um assunto importante para o desenvolvimento de estirpes de panificação mais adequadas ao processo de congelamento de massas panares. Num trabalho anterior, foi demonstrado que a resistência ao congelamento das estirpes de *T. delbrueckii* aqui em estudo, poderia ser atribuída à sua maior capacidade de preservação da integridade da membrana. Em *S. cerevisiae*, uma diminuição da temperatura induz a expressão de vários genes, alguns dos quais originam um fenótipo de sensibilidade ao frio quando removidos. No entanto, pouco se sabe acerca do papel desempenhado por muitos destes genes e dos mecanismos reguladores que controlam esta resposta. O gene *HSP12* é um desses genes. Além disso, a proteína Hsp12 parece estar localizada na membrana plasmática o que a torna uma boa candidata para um papel na preservação da integridade membranar durante o congelamento. O Capítulo 6 descreve as respostas ao frio do mutante Δ*hsp12*, dando ênfase ao contributo da proteína Hsp12p na tolerância ao congelamento e à sua relação com a trealose. Os resultados demonstram que a proteína Hsp12 desempenha um papel na crioresistência apesar do mutante nulo no gene *hsp12* se ter revelado mais resistente ao congelamento do que a estirpe selvagem. De facto, a estirpe mutante Δ*hsp12*, apresentou uma concentração intracelular de trealose

mais elevada quando comparada com a estirpe selvagem, o que parece justificar a sua maior crioresistência. A sobreexpressão do gene *HSP12* na estirpe Δ*tps1* (que não tem capacidade de acumular trealose) revelou um claro aumento na tolerância ao congelamento bem como na resposta ao stress induzido pelo aumento da temperatura. Apesar do mutante Δ*hsp12* apresentar uma maior acumulação de trealose em fase estacionária, esta acumulação encontra-se diminuída em resposta ao choque térmico, parecendo indicar que a proteína Hsp12 tem também um papel na resistência a temperaturas elevadas.

A utilização de leveduras na indústria de panificação requer um conhecimento aprofundado da sua ecologia, fisiologia, bioquímica e biologia molecular. O trabalho desenvolvido no âmbito desta tese pretendeu contribuir para este conhecimento e para o fornecimento de novas estratégias de biologia molecular para a identificação e caracterização destas leveduras, abrindo novas possibilidades para a inovação em leveduras de panificação.

Index

Abbreviation list

ALP	Alkaline Phosphatase
APS	Ammonium Persulfate
dH_2O	Deionized H2O
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
GDP	Glyceraldehyde-3-Phosphate Dehydrogenase
GSR	General Stress Response
HPLC	High Performance Liquid Chromatography
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP	Heat Shock Protein
HSR	Heat Shock Response
HXT	Hexose Transporter
IGT1	Intermediate Glucose Transporter
IPTG	Isopropylthio-ß-D-Galactoside
ITS	Internal Transcribed Spacers
LB	Luria Bertani
LGT1	Low Glucose Transporter
MFS	Major Facilitator Superfamily
OD	Optical Density
ON	Overnight
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
Rpm	Rotation per minute
SDS	Sodium Dodecyl Sulfate
sHSP	Small Heat Shock Protein
ssDNA	Single strand DNA
STRE	Stress Response Elements
TCA	Thrichloroacetic Acid
TEMED	1,2-bis-(dimethylamino)-Ethane
TPS1	Trehalose-6-Phosphate Synthase
w/o	Without
wt	Wild type

Amino acids abbreviations:

Alanine Ala A
Arginine Arg R
Asparagine Asn N
Aspartic acid Asp D
Cysteine Cys C
Glutamic acid Glu E
Glutamine Gln Q
Glycine Gly G
Histidine His H
Isoleucine Ile I
Leucine Leu L
Lysine Lys K
Methionine Met M
Phenylalanine Phe F
Proline Pro P
Serine Ser S
Threonine Thr T
Tryptophan Trp W
Tyrosine Tyr Y
Valine Val V

Species abbreviations:

A. nidulans	*Aspergillus nidulans*
C. albicans	*Candida albicans*
E. coli	*Escherichia coli*
H. polymorpha	*Hansenula polymorpha*
K. lactis	*Kluyveromyces lactis*
P. stipitis	*Pichia stipitis*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pastorianus	*Saccharomyces pastorianus*
Sch. pombe	*Schizosaccharomyces pombe*
T. delbrueckii	*Torulaspura delbrueckii*
Z. bailli	*Zygosaccharomyces bailli*

Chapter 1

General Introduction

Baking History - A taste for the Past

The history of yeast association with human society is synonymous with the evolution of bread, beer and wine. References to winemaking date back to 5,000 BC, when yeasts were accidentally used in spontaneous fermentations in Egypt (Samuel, 1996) and Phoenicia, but historians believe wine production probably occurred much earlier than this (Robinson, 2006). Also ancient bread makers probably relied on spontaneous fermentations to leaven their doughs and may have developed sourdoughs at an early date by reserving a portion of a fermented sponge to inoculate the next day's batch (Wirtz, 2003). Archeological evidence of a 'brewery' dating back to about 1,500 BC was discovered beneath the Sun Temple of Queen Nefertiti, suggesting that beer was produced on an industrial scale in ancient Egypt (Fleet, 2006). The microbial science of these products started in the mid-1600s with the first observations of yeast cells being described by Antonie van Leeuwenhoek (The Netherlands). The effects of these findings stayed latent until the studies of Pasteur (France) and Hansen (Denmark) during 1850–1900. The role of micro-organisms in fermentation was first identified in 1876 when Louis Pasteur proved that fermentation was due to living cells. Selection of yeasts with desirable properties was presumably ongoing from these early times but it was not until 1881 that Emil Hansen isolated the first pure yeast culture from the Carlsberg Brewery, a condition for the systematic selection and improvement of strains (Fleet, 2006; Wirtz, 2003).

Baker's yeast: important qualities for baking applications

In the history of human nutrition, a diversity of bakery products has been created and continues today. Bread is made mostly from flour dough that is allowed to raise (leaven) before baking in the oven. The making of bread requires three main ingredients: flour, water, and yeast. The yeast main role in the bread making process is to promptly ferment the sugars available in the flour of the dough or that are being added to the dough. As result from an efficient fermentation, the yeast produces CO_2 and ethanol; the carbon dioxide is trapped within the gluten matrix of the dough causing the leavening or rising, while the ethanol contributes to flavor creation, together with other volatiles compounds and flavors precursors that are formed during the fermentation process. Technically, the most important properties of baker's yeast comprise (1) leavening ability in the dough; (2) the ability to adapt to different carbon sources, by expressing invertase and maltase activities (see Chapter 3); and (3) stress resistance, particularly osmo- and cryo-tolerance (see Chapter 6). Obviously yeast should contribute to the flavor of the baked products as well and grow fast in molasses from where they are commercially produced. Commercial baker's yeasts are domesticated strains essentially of *Saccharomyces cerevisiae* that have been selected and optimized for baking applications. These particular features are the result of natural adaptation among with the continuous selective pressure made by yeast manufacturers for many years. However, it is still important to improve some parameters that are far from optimal. Fermentative capacity is one of the most important bio-technological challenges in baking industry. Yeast's gassing rate is crucial in baking technology and

it depends mostly on characteristics of baker's yeast. Also tolerance to different stresses, like osmotic or freezing is clearly insufficient. When baker's yeast is subjected to osmotic stress cells rapidly dehydrate which limits growth and gas-production capacity (Attfield, 1997; Randez-Gil *et al.*, 1999). Consequently, proofing time (period where the yeast is allowed to leaven or raise the dough) increases and the bread volume is reduced. In frozen-sweet dough, freezing and thawing further reduce the water activity aggravating this situation. Furthermore, freezing and frozen storage of dough has a negative impact on the baking performance due to cell damage (Attfield, 1997; Randez-Gil *et al.*, 1999). Therefore, the development of yeast strains with better gassing power in frozen and frozen-sweet dough is of great economic interest.

Sugar metabolism by bakers' yeast

Nowadays physiological requirements on baker's yeast between production and application represent an apparent contradiction (fig.1). In fact, sugar-limited respirofermentative fed batch cultivation (yeast production phase) is implied to render a yeast product that has developed a high fermentative capacity although this requirement is not important during this phase. Subsequently the gassing capacity (fermentation) is used in the application phase in the dough, under anaerobic, sugar excess conditions. Therefore an exceptional physiological flexibility is required for baker's yeast.

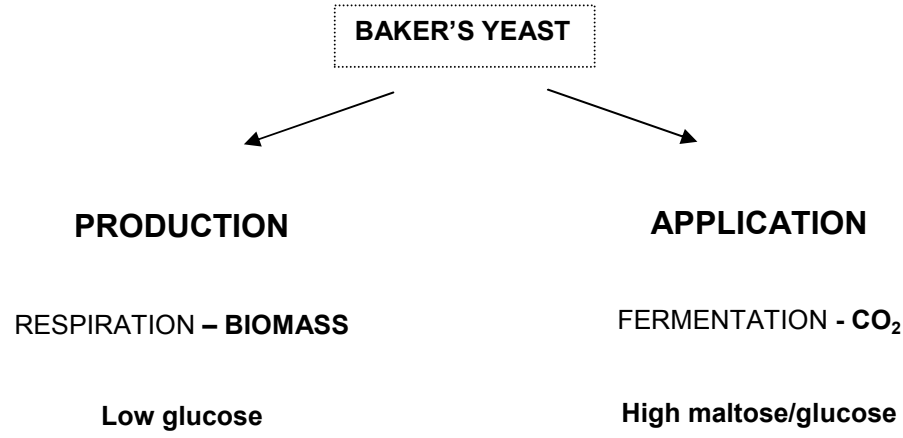

Fig.1. The paradox of baker's yeast production and application. Baker's yeast is produced in sugar-limited, respiro-fermentative fed batch cultivations, and under these conditions should develop the potential to readily ferment excess sugar to CO_2 (and ethanol) in subsequent dough application. Hence, under respiratory physiological conditions where metabolic flux is toward cell growth and biomass production, yeast is expected to develop a good fermentative capacity. Adapted from Functional Genetics of Industrial Yeasts (Topics in Current Genetics)(Winde, 2003).

Molasses

Further than good fermentative capacities, high freezing and osmotic tolerance, another main quality must be considered when selecting a yeast strain for the baking industry (Benitez, 1996): effective biomass production in molasses. Because it is cheap and easily available and contains some nitrogen and several vitamins and minerals necessary for yeast growth, molasses are the main substrate used for large-scale baker's yeast production. However, molasses are considered a major factor of variation in the quality of baker's yeast (Sinda E. & Parkkinen, 1979). These substrates are highly variable, and contain different proportions of sugars where sucrose is the major part but there is also a quite high amount of glucose and fructose. Sucrose is cleaved outside the cell by invertase into glucose and fructose. Invertase is also capable of cleaving raffinose, a trisaccharide also present in molasses, into fructose and melibiose (glucose-galactose), but melibiose is generally not assimilated (Vaughan-Martini & Martini, 1998) (fig.2).

Bread dough

The main fermentable sugar in bread dough is maltose, liberated from starch by amylase activity (*α*-glucosidase) in flour. This disaccharide is transported through a maltose permease and is subsequently hydrolyzed into glucose by maltase (fig.2). The order in which these different carbohydrates are fermented by *S. cerevisiae is* not random, but rather is based on a specific hierarchy, with glucose being the preferred sugar. Consequently, in dough containing glucose, sucrose, and maltose, the disaccharides will be fermented only when the glucose is consumed. The monosaccharides glucose, fructose and mannose, are transported into cells by hexose transporter (Hxt) proteins across the plasma membrane, and subsequently are further metabolized in glicolysis (fig.2). The endpoint of glicolysis is pyruvate, and it depends on the growth condition whether pyruvate is used for respiration or fermentation. When cells grow in an anaerobic environment, sugars are exclusively fermented into CO_2 and ethanol. However, cells are able to use glucose for fermentation also when grown aerobically if glucose is presence at high concentrations. This phenomenon is generally refered as the Crabtree effect (Crabtree, 1929; De Deken, 1966). In an aerobic environment, sugars are completely used through respiration only at low growth rates in the presence of low carbon source concentrations. Glucose further than being the favorite carbon source for yeast, operates different mechanisms that act at many levels, assuring its primary utilization and the regulation of metabolism, cell growth, and development. For the most part, regulation is mediated by catabolite repression, acting at early steps in various catabolic pathways. The aim of the regulation is to induce utilization of most favoured carbon source (glucose), and to exclude utilization of other carbon sources if sufficient amount of glucose is available. Therefore, in most strains of *S. cerevisiae,* glucose represses genes responsible for maltose transport and hydrolysis, as well as the invertase that hydrolyzes sucrose to glucose and fructose. Expression of invertase is repressed by high glucose concentrations (Mormeneo & Sentandreu, 1982). Although the high levels of invertase activity required for

growth in molasses, there is evidence that the capacity of *S. cerevisiae* to ferment high sucrose concentrations, like those present in sweet bread doughs, is inversely related to the activity of this enzyme (Attfield & Kletsas, 2000).

The study of sugar utilization patterns, maltase and invertase activities, sugar uptake rates and respiration/fermentation rates contributes to a better understanding of the mechanisms underlying some of the most important characteristics of a good baker's yeast. In order to better evaluate the potential offered by *T. delbrueckii* to the baking industry we have carried out physiological and biochemical studies of this yeast in batch cultures with sugars frequently found in molasses and in bread dough, using them alone and in mixtures (see Chapter 3).

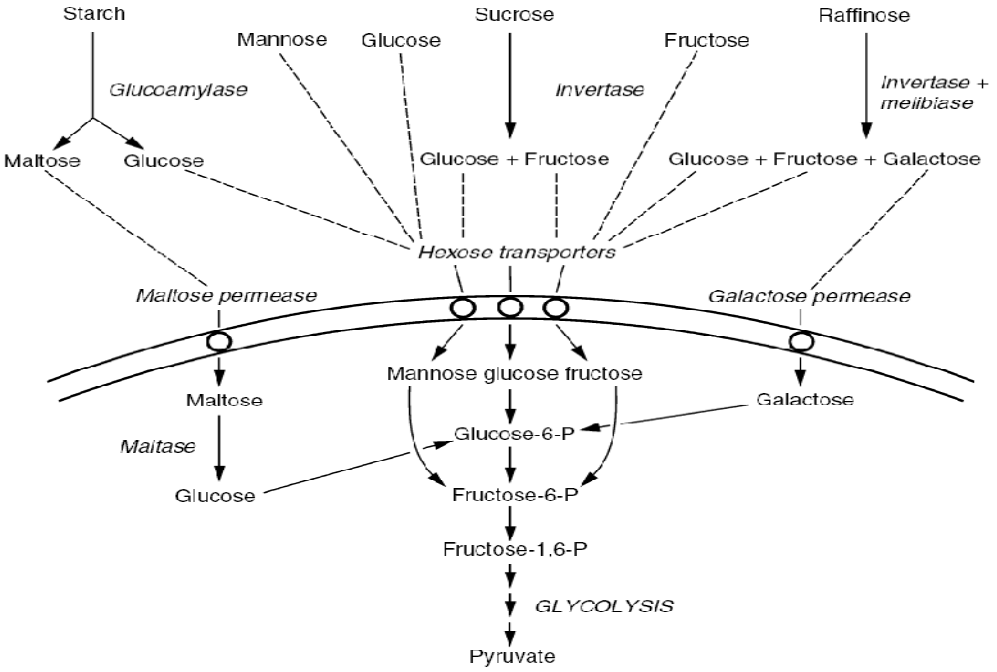

Fig. 2. Schematic outline of the transport and first steps in the carbohydrate metabolism of yeasts. See text for details. (Modified from Handbook of Food Spoilage Yeast, 2003) (Deák, 2003)

Sugar transport in yeast

Sugar transporters

The sugar porter family is the largest within the major facilitator superfamily (MFS), which includes proteins from Bacteria, Achaea and Eukarya, with very diverse sequence and function (Baldwin & Henderson, 1989; Henderson & Maiden, 1990; Maiden *et al.*, 1987). Proteins belonging to the MFS exhibit highly structural conservation, however they share little sequence similarity (Vardy *et al.*, 2004). Generally, these permeases have 12 putative transmembrane segments, consisting in a single integral membrane protein with two sets of six hydrophobic transmembrane-spanning (TMS) α-helices connected by a

hydrophilic loop, whose amino- and carboxy-terminal regions are localized in the cytoplasm (Kruckeberg, 1996; Pao *et al.*, 1998; Saier, 2000). The strong similarity between the two sets of hydrophobic TMS of MFS proteins together with their structure supports the theory that they result from a gene duplication event that probably took place before de divergence of MFS families (Maiden *et al.*, 1987; Pao *et al.*, 1998). Sugar transport across the plasma membrane is the first and obligatory step of its utilization. Yeasts can use different carbon sources for growth but evolution has selected mechanisms for the preferential utilization of glucose. Permeability of biological membranes is quite restricted, demanding that most of the cellular nutrients enter the cell via specific transport systems. Both, facilitated diffusion and proton-symport transport systems for sugars have been described in yeasts. In facilitated diffusion, solutes are transported down a concentration gradient, by a uniport mechanism, and a facilitated transporter can saturate when the intracellular chemical potential of a component reaches the same value as that in the medium. Once this happens, no further increase in the rate of transport can occur, since the binding site on the transporter is occupied essentially all the time. Transport by facilitated diffusion is passive and energy independent (Lagunas, 1993). The driving force for this process is the electrochemical gradient of the transported solute (van Dam & Jansen, 1991).

In active transport, molecules move against this gradient, that is, from an area of low concentration to an area of high concentration of the solute. There are two types of active transport: primary and secondary. In primary active transport, the cell uses directly the energy that it gets from the hydrolysis of ATP, from radiant energy, or from electron transport. Examples of proteins that transport molecules across a cell membrane against the concentration gradient are the Na+-K+ pump, which can transport glycoproteins and many drugs across the cell membrane. Secondary active transport use accumulated energy of an electrochemical gradient to transport molecules against their concentration gradient, coupled with the simultaneous movement of another molecule (normally H^+ or Na^+) in the same (symport) or opposite (antiport) direction (Lodish, 1995). Such a mechanism becomes central during growth at very low extracellular sugar concentrations when an intracellular accumulation of hexoses may be necessary to allow the hexose kinases to function optimally. Evidently, it may appear that yeast species possessing proton-hexose symport systems are better adapted to grow at low hexose concentrations (Postma *et al.*, 1989; van Urk *et al.*, 1989). However, since a facilitated diffusion transport system is most efficient only under reasonably constant levels of the carrier substrate, apparently such a system might not be appropriate for yeasts like *S. cerevisiae*. This yeast which uptakes hexoses only by facilitated diffusion, has clearly worked out this setback by developing an unusual diversity of hexose transporter proteins (Hxtp) with specific individual properties and kinetics (Boles & Hollenberg, 1997).

Hexose transport

Hexose transport in *S. cerevisiae*

Hexose carriers have been characterized genetically in *S. cerevisiae.* Among about 5600

protein-coding genes in *S. cerevisiae*, at least 271 encode for predicted or established permeases (Van Belle & Andre, 2001). Twenty of them encode hexose transporters and related proteins, the so-called *HXT* gene family (Boles & Hollenberg, 1997; Kruckeberg, 1996). Of these, only *HXT1–HXT7* encode transporters that are important for growth and metabolism of glucose (Diderich *et al.*, 1999a; Reifenberger *et al.*, 1995). The galactose transporter, encoded by *GAL2*, is also a member of the *HXT* gene family (Nehlin *et al.*, 1989; Szkutnicka *et al.*, 1989). Two members of the family, encoded by *SNF3* and *RGT2*, have lost the ability to transport hexoses; instead they function as sensors of the extracellular glucose concentration. This glucose signal is involved in regulating the expression of various *HXT* genes (Ozcan & Johnston, 1999). The remaining members of the family (*HXT8–HXT17*) are phenotypically silent, and may not be expressed under normal physiological conditions (Diderich *et al.*, 1999a). As discussed by Wieczorke *et al* (1999), the large number of hexose transporter proteins in baker's yeast seems to reflect its adaptation to the variety of environmental conditions to which yeast cells are exposed. Transporters catalyze uptake of solutes and while doing so they undergo some conformational change, thus showing specific binding of the substrates. Two kinetically distinct glucose uptake systems have been initially described in *S. cerevisiae*: a constitutive low-affinity system (K_m=15–20 mM) and a glucose-repressible high-affinity system (K_m=1–2 mM) (reviewed by (Ozcan & Johnston, 1999)). These two components are now considered to consist of several different transporters contributing to overall kinetic properties of the systems. The affinity for glucose of the major Hxt proteins was determined by individual expression of these transporters in a *hxt* null strain (Reifenberger *et al.*, 1997),and differs significantly; for example, Hxt1 and Hxt3 have a low-affinity for glucose, whereas Hxt2, Hxt6, and Hxt7 have a high-affinity (Reifenberger *et al.*, 1997). The low-affinity transporters are expressed at high glucose concentrations, whereas the high-affinity transporters are expressed at low glucose concentrations (Diderich *et al.*, 1999b). It should however be noted that most of the data on the kinetics of glucose transport was obtained from individual expression of *HXT* genes in an *hxt* null mutant, so that the results may not reflect the *in vivo* functions of the Hxt proteins. A single Hxt protein might behave differently in terms of affinity, which may be modulated by means of interaction between different Hxt proteins. Furthermore, the missing *HXT* genes may be important for regulation of expression of other *HXT* genes (Meijer *et al.*, 1998; Ozcan & Johnston, 1999).

Hexose transport in non-*Saccharomyces* yeasts and filamentous fungi

The number of hexose transporters among yeast is very variable, ranging, for instance, from 20 hexose transporters in *S. cerevisiae* and *Candida albicans,* to six in *Schizosaccharomyces pombe* or seven in *Kluyveromyces lactis. In silico* analysis of *K. lactis* genome (De Hertogh *et al.*, 2006) showed that this yeast has 20 sugar transporter genes, still only seven of them have been characterized.
Based on protein sequence homology (TBLAST search) with CaHg1, the first gene encoding a glucose transporter in *C. albicans* (Varma *et al.*, 2000)*,* another 19 putative glucose transporters designated Hgt2-Hgt20 was revealed (Arnaud *et al.*, 2007; Fan *et al.*, 2002). In the fission yeast *Sch. pombe* a family of six hexose transporter genes (*GHT1-GHT6*) has been identified (Heiland *et al.*, 2000). These six transporters show high similarity at the nucleotide and amino acid level. In *Pichia stipitis* three genes

encoding glucose transporters *SUT1*, *SUT2* and *SUT3* (sugar transporters) have been identified, which probably constitute only a subfamily of glucose transporters (Weierstall *et al.*, 1999). Just recently with the complete sequencing of *P. stipitis* genome the occurrence of several more putative sugar transporter genes was revealed (Jeffries *et al.*, 2007). Stasyk *et al*. 2008 described Hxt1, the first functional hexose transporter identified in the methylotrophic yeast *Hansenula polymorpha* (Stasyk *et al.*, 2008). Wei *et al.* 2004 found at least 17 putative hexose transporters in the genome of *Aspergillus nidulans (Wei et al., 2004).* So far only one hexose (particularly fructose) transporter has been described for *S. pastorianus* (Gonçalves *et al.*, 2000) and in *Zygosaccharomyces bailii (Pina et al., 2004)*. Fsy1 (fructose symport) is a specific fructose/H+ symporter, which mediates high-affinity fructose uptake (it does not transport glucose) in *S. pastorianus* (Gonçalves *et al.*, 2000). In *Z. bailii* Ffz1 (fructose facilitator of *Zygosaccharomyces)* does not accept glucose as substrate and displays low affinity for fructose (Pina *et al.*, 2004).

Hexose transport in *Torulaspora delbrueckii*

Two natural habitats of *T. delbrueckii* are bread dough's and fruit juices, such as grapes, environments that are rich in sugars. As a consequence of growth and fermentation of these sugars, the yeast experiences dramatic changes in its physicochemical environment, and to sustain its growth it must adapt to these changes. The sugar concentration may decline from 1 M to 10^{-5} M during fermentation, and the overall composition of the medium will be altered by yeast metabolism. The sugar transport activity of the cell and the proteins that mediate sugar transport must be responsive to these changing conditions, thus the capacity and kinetic complexity of hexose transport in the yeast may be reflected in a large number of sugar transporter genes in its genome. Based on this assumption the probable existence of multiple hexose transporters with different affinities for glucose in *T. delbrueckii* is not surprising. Previous work has demonstrated that this yeast displays a mediated glucose transport activity best fitted assuming a biphasic Michaelis–Menten kinetics with a low- (apparent K_m = 8.32 ± 0.55 mM) and a high-affinity component (apparent K_m = 1.30 ± 0.34 mM) (Alves-Araújo *et al.*, 2005). Until now, just one glucose transporter has been identified in *T. delbrueckii*, the low-affinity glucose transporter *LGT1* (Alves-Araújo *et al.*, 2005). Lgt1p when expressed in a *hxt* null strain of *Saccharomyces cerevisiae* presents an apparent K_m value of 36.5 ± 3.1 mM, in the range of the low-affinity component, and a V_{max} of 1.1 ± 0.04 nmol/s/mg dry wt. This transporter is also able to mediate significant fructose uptake in the *hxt* mutant, although with a lower affinity than for glucose, apparent K_m value of 51.4 ±3.0 mM. The impact of *LGT1* disruption to the overall of sugar transport of this yeast is reported in Chapter 5. It is also likely that *T. delbrueckii* possesses high-affinity transporters, a proposal supported by the kinetics of glucose transport that assumes biphasic Michaelis–Menten kinetics. These results together with our first assumption suggest the existence of other physiological relevant glucose transporters, besides Lgt1p. Therefore one aim of this work was to screen by different approaches, for other genes involved on glucose transport in *T. delbrueckii*. Consequently, it is reported the existence of a second glucose transporter *IGT1* in *T. delbrueckii*, the first of intermediate affinity (see Chapter 5).

Baker's yeast and stress resistance

In baking industry, yeasts encounter numerous stresses. During production, yeasts must adapt to low sugar and high aeration, repressing fermentation to produce large amounts of biomass. Cells are then perserved in cold, frozen or dry state until use, when rehydration or thawing and inoculation cause osmotic shock in a new environment that requires the induction of enzymes for maltose utilization under semianaerobic conditions. The low stress resistance during active fermentation of yeast is disadvantageous for its use in industrial applications, and it would be highly advantageous to have yeast strains that do not lose their stress resistance during fermentation (Attfield, 1997). Furthermore, human food habits have changed in the past few years, increasing frozen products demand. Yeasts are incapable to deal with such stresses exhibiting reduced fermentation performance and compromising product quality (Attfield, 1997; Ivorra *et al.*, 1999; Pretorius, 2000; Verstrepen *et al.*, 2004). Consequently, these effects have a great technological and economic impact in baking industry. Undoubtedly, the ability of baker's yeast to cope with stress conditions is an essential physiological requirement in baking industry.

Cryoresistance in baker's yeast

A high level of tolerance to freeze-thawing in yeasts is advantageous for the increasingly current use of frozen dough for bread production. Reduced yeast vitality after freezing and thawing causes loss of fermentation capacity and makes it necessary to use a larger amount of yeast, leading to longer proofing times (i.e., the resting period after mixing during which fermentation takes place) and reduced product volume (Teunissen *et al.*, 2002). Thus, bread-making industry holds a high demand for yeast strains with improved freeze resistance. In this work, we have focused our attention on the study of mechanism underlying freezing tolerance in yeast with possible implications in improvement of stress resistance (see Chapter 6). Our interest in freeze tolerant yeasts for baking was also on the basis of the choice to elucidate several aspects of the metabolism and fermentative behavior of the cryoresistant yeast *T. delbrueckii* (see Chapters 3 and 5), reported to be more freeze-tolerant than *S. cerevisiae* and with potential application in baking industry (Alves-Araújo *et al.*, 2004a).

Frozen storage and effects of freezing on baker's yeast

One of the first stresses encountered by baker's yeast cells during preparation of frozen dough is the decrease, after mixing, in the environment temperature, known as cold-shock. This change impairs the correct functioning of both the membrane and the translational apparatus as result of reduced membrane fluidity and stabilization of the secondary structures of DNA and RNA (Inouye, 1999; Thieringer *et al.*, 1998). While positive cold temperatures lead to the synthesis of specific proteins associated with the development of transient phenotypic adaptation (Rodriguez-Vargas *et al.*, 2002; Sahara *et al.*, 2002), freezing is frequently a lethal stress to cells. At subzero temperatures, the damaging

effects on yeast cells depend on the freezing rate. At rapid freezing, cells are injured by the formation of intracellular ice crystals, which leads to membrane disruption (Morris *et al.*, 1988). Structural examination of these cells shows discontinuous nuclear membranes, disappearance of vacuoles and DNA spread all over the cells (Kaul *et al.*, 1992). On the other hand, in cells exposed to low freezing rates, an osmotic shrinkage of the cells and frozen extracellular water is observed. Therein, cells become exposed to hyperosmotic solutions and try to balance that by moving water across the membranes (Wolfe & Bryant, 1999). In this case the cells suffer cellular damages similar to the ones caused by dehidratation. During frozen storage, ice crystals growth can further deteriorate the plasma membrane and damage activity of different cellular systems. All together, these findings indicate that freezing is a very complex stress, in which different stresses and stress responses appear to play important roles. Therefore, freezing tolerance is likely to involve different mechanisms working in concert.

Yeast stress response

Overview

The response and adaptation mechanisms to stress are highly complex. Hence, research on stress responses, particularly in times of global gene and protein expression analyses, frequently comprises almost all aspects of cell biology. Cellular response to stress is obviously aimed at shielding cells from the harmful effects of stress and at repairing possible damage.

Yeast strains used for brewing, baking and winemaking are intrinsically tolerant to arrange of extreme conditions. This tolerance is presumably acquired by rapid molecular responses that protect against damage caused by ongoing exposure to the same or other forms of stress. These responses include changes in gene transcription, translational and post-translational modifications of stress-associated protein, and are triggered, at least in part, by stress-induced denaturation of proteins, disordering of membranes, DNA damage and metabolic disturbances (Mager & Ferreira, 1993; Piper, 1993; Siderius *et al.*, 1997).Tolerance to stress is acquired by means of protective biochemical processes which include the synthesis of osmolytes (e.g. glycerol), trehalose, heat shock proteins (HSPs), increased chaperone activity, enhanced radical oxygen scavenging, changes in redox control, increased proton pumping activity, adjustments in carbon/nitrogen balance and altered ion and water uptake (Estruch, 2000; Parrou & Francois, 1997; Yale & Bohnert, 2001). These stress response mechanisms not only start the repair of macromolecular damage caused by stress but most likely also establish a tolerant state, which helps prevent further damage. Stress responsive genes that are part of the general stress response machinery of yeast are presumed to encode proteins with functions that are necessary to cope with damage under various stress conditions. The expression of genes is controlled by specific regulatory factors up-stream of each gene. In *S. cerevisiae* there are two major independent stress responses: the general stress response (GSR) and heat shock response (HSR). The general stress

response is induced by a wide variety of stressing agents including heat, osmotic stress, oxidative stress, nitrogen starvation, ethanol, sorbate and low pH (Chatterjee *et al.*, 2000; Ruis & Schuller, 1995).

Transcription factors that are required to induce or repress stress regulated genes are well-characterized components of the stress response. These include transcription factors that are specific for the heat shock response, such as the HSF (Hsf1) that binds to a specific conserved promoter sequence, the heat shock element (HSE) (Boy-Marcotte *et al.*, 1999; Eastmond & Nelson, 2006), or transcription factors that respond to a variety of cellular and environmental stress conditions, such as the Msn2 and Msn4 transcription factors that binds to stress-response element (STRE) (Berry & Gasch, 2008; Eastmond & Nelson, 2006; Izawa *et al.*, 2007).

A quick view on heat shock response

The heat shock response is characterized by the rapid expression of a unique set of proteins collectively known as heat shock proteins (HSPs) (Kregel, 2002; Ritossa, 1996). These highly conserved proteins are ubiquitous proteins found throughout all kingdoms. Whereas classically described as a response to thermal stress (hence the term heat shock) (F. Ritossa, 1962; Gerner & Schneider, 1975), heat shock proteins can also be induced when yeast cells are exposed to other different stresses (Kobayashi & McEntee, 1990; Motshwene *et al.*, 2004; Sales *et al.*, 2000; Susek & Lindquist, 1990), entailing that they should more correctly be referred to as stress proteins. Several Hsps contribute to yeast thermotolerance by acting as molecular “chaperones” to prevent protein aggregation (Morano *et al.*, 1998) and support proteolysis of stress-damaged proteins.

The cold and freeze response

Adaptation of yeast cells to a downshift in temperature occurs by alterations in metabolic rate, ion concentration, membrane composition, and by change in gene expression. Acquisition of stress tolerance is possible for almost all stress conditions and is considered to be one of the main purposes of the cellular stress response. *Geotrichum candidum* adapted to freeze-thaw stress by pre-treatment at chill temperatures (Thammavongs *et al.*, 2000). Kandror *et al*. 2004 showed that below 10 ºC, yeast show an adaptive response that protects viability to subsequent exposure to low or freezing temperatures (Kandror *et al.*, 2004). Probably this is mainly due to the strong accumulation of trehalose and molecular chaperones such as heat shock proteins, such as Hsp104, Hsp42 and Hsp12 (Kandror *et al.*, 2004) (discussed later in this Chapter). More recently it was shown that cells of industrial strains growing at 15 ºC displayed enhanced freeze and frozen-storage resistance than those grown at 30 ºC (Rodriguez-Vargas *et al.*, 2007), supporting that death during freezing can be prevented or decreased by a previous growth at low temperatures.

Thus, a well-adapted metabolism at low temperatures appears to induce higher resistance to low temperature and freezing stress. Therefore, it is valuable to study genes reported as cold

induced or essential for growth at cold temperatures. In this course, we report the investigation on Hsp12p, described to protect membranes from desiccation as well as to be induced at 0 ºC as part of the near-freezing response (Kandror *et al.*, 2004).

Heat shock protein Hsp12p

Hsp12p was reported for the first time in 1990 as a 15kDa protein that was synthesized at a low level during growth on glucose and was induced upon glucose deprivation, and consequently named *GLP1* for glucose lipid regulated protein (Stone *et al.*, 1990).

In 1998 Hsp12p was described as a late embryogenic abundant (LEA)-like protein, since both Hsp12p and LEA proteins are synthesized in response to desiccation stress. A role in water replacement has also been ascribed for this 12 kDa hydrophilic stress response protein, which has been shown to be located close to the plasma membrane (Sales *et al.*, 2000) and at the cell wall (Motshwene *et al.*, 2004). Sales *et al*. (2000) also demonstrated that Hsp12p protects liposomes against ethanol-induced lysis as well as against desiccation and subsequent rehydration, in a manner analogous to trehalose. *HSP12* has also been shown to be up-regulated in response to cell-wall-distressing agents (Karreman *et al.*, 2007), suggesting that Hsp12p may play a function in the cell wall. Also the volume of Δ*hsp12* cells were less affected by changes in external osmolarity than that of wild-type cells, suggesting that Hsp12p might act in maintaining cell wall flexibility (Motshwene *et al.*, 2004). Recent studies using atomic force spectroscopy showed that Hsp12p does indeed act as a cell wall plasticizer *in vitro* (Karreman *et al.*, 2007). The gel strength of agarose, used as a model system to represent the β-glucan layer of the cell wall, increased upon incorporation of solutes known to upregulate *HSP12*, an effect that could be reversed upon simultaneous incorporation of Hsp12p (Karreman & Lindsey, 2005). Factors that influence *HSP12* induction include heat and cold shock (Kandror *et al.*, 2004; Praekelt & Meacock, 1990), water scarcity stress or desiccation (Garay-Arroyo *et al.*, 2000), agents affecting the cell wall integrity (Karreman *et al.*, 2007), salt stress (Varela *et al.*, 1995), osmotic stress, entry into stationary phase, glucose limitation, nutrient depletion (Praekelt & Meacock, 1990), oxidative stress (Kitagawa *et al.*, 2005; van Bakel *et al.*, 2005), as well as the presence of mannitol (Mtwisha *et al.*, 1998) or ethanol in the growth medium (Piper, 1995).

Mild stress and cross-stress protection responses

Yeast cells exposed to mild stress can develop tolerance not only to higher levels of the same stress, but also to stress caused by other agents. This phenomenon is called cross-protection and is caused by the expression of general stress-responsive genes under mild stress conditions (Berry & Gasch, 2008; Chen *et al.*, 2003; Smith *et al.*, 2004). This cross-protection implies that different stress conditions involve common cellular responses, such as adjustment of energy metabolism and production of protective proteins (such as heat shock proteins) or small protective molecules (compatible solutes such as glycerol or trehalose).

For example, a brief temperature shock not only increases yeast thermo-tolerance, but may also increase tolerance to other stressors such as ethanol (Costa *et al.*, 1993), a high salt concentration and oxidative stress (Lewis *et al.*, 1995). In 1994 Steels *et al*. investigated the relationship between yeast tolerance to heat and oxidative stress, and found that a mild heat shock induced tolerance to an otherwise lethal temperature and H_2O_2 stress (Steels *et al.*, 1994). Similarly, pre-treatment of yeast cells with a mild osmotic shock conferred increased resistance to heat shock (Varela *et al.*, 1992) and the exposure of yeast to ethanol, sorbic acid and low external pH induced greater thermotolerance (Coote *et al.*, 1991; Plesset *et al.*, 1982) (for cross-protection with trehalose see Chapter 6). The phenomenon of cross-protection is consistent with the yeast cellular responses that not only initiate the repair of macromolecular damage caused by stress but presumably also establish a tolerant state, which helps prevent further damage. Stress responsive genes that are part of the general stress response machinery of yeast are presumed to encode proteins with functions that are necessary to cope with damage under various stress conditions.

Trehalose in stress tolerance

In freeze-thaw stress a large extent of the damage caused to cells results from freezing rather than from thawing and mainly due to physical effects, for instance ice crystal formation and dehydration, as referred above (Park *et al.*, 1997). Trehalose is a non-reducing disaccharide whose presence is a typical hallmark of yeast cells rapidly adapting to changing environmental conditions. It plays an interesting dual role as a storage carbohydrate and as a stress protector (François & Parrou, 2001). Trehalose functions as a thermoprotectant and a cryoprotectant by stabilizing cell membranes and accumulates markedly in cells exposed to a non-lethal heat shock.

In *Saccharomyces cerevisiae*, the trehalose content and the level of stress resistance of cells adjusts as a function of environmental conditions and undergoes drastic changes during the life cycle. The level of trehalose is very low in yeast cells growing exponentially and it increases when cells enter the stationary phase or undergo stressing conditions (Thevelein, 1984). The accumulation of trehalose appears to be associated with periods of reduced growth, such as when cells are starved of nitrogen, phosphate or sulphur, as well as during the stationary phase of growth on glucose (Lillie & Pringle, 1980).

Accumulation of trehalose increases stress resistance in yeast growing on non-fermentable carbon sources (Van Dijck *et al.*, 1995), resistance to freezing (Soto *et al.*, 1999) and resistance of endocytosis to ethanol (Lucero *et al.*, 2000). Trehalose may also protect membranes from desiccation to maintain membrane integrity by substituting water molecules and binding to the polar head-groups of phospholipids (Crowe, 1992; Sano *et al.*, 1999). It also protects yeast cells and cellular proteins from damage caused by H_2O_2 (Benaroudj *et al.*, 2001), reduces intracellular ROS concentration and decreases *in vivo* lipid oxidation during exposure to menadione (Herdeiro *et al.*, 2006).

In these conditions, dramatic changes in cell viability are accompanied by the aggregation of macromolecules and the denaturation of proteins. It seems likely that trehalose reduces protein

aggregation and maintains polypeptide chains in a partially folded state, thus increasing tolerance to thermal and desiccation stresses (Singer & Lindquist, 1998; Thevelein, 1984; Wiemken, 1990). Even when the intracellular trehalose content is low it protects cells against severe heat shock and glucose starvation (Plourde-Owobi *et al.*, 2000).

Trehalose biosynthesis

Cellular levels of trehalose are controlled by a balance between synthesis and degradation. The biosynthesis of trehalose is catalysed by a two-step process involving trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase on a multimeric protein complex (see fig.3). This complex consists of four different subunits encoded by *TPS1, TPS2*, which catalyze the reactions of trehalose biosynthesis, and *TSL1* and *TPS3*, which have no catalytic activity but which stabilize the trehalose synthase complex (Bell *et al.*, 1998; Vuorio *et al.*, 1993). The complete enzyme consists of Tps1p, Tps2p, Tps3p and Tsl1p, which act as both synthase and phosphatase. Deleting *TPS1*, the gene that encodes trehalose-6-phosphate synthase, results in loss of cell ability to synthesize trehalose or grow on glucose. Although all genes encoding subunits of the trehalose synthase complex display significant sequence homology to *TPS1* gene, none of the other subunits replaces the function of Tps1p in synthesizing Tre6P or in controlling glucose influx into glicolysis (Bell *et al.*, 1998). The Δ*tps1* mutant will play a central role in Chapter 6.

Trehalose degradation

Yeasts have two types of trehalases, known as acid and neutral trehalases because of their optimal activity pH (4.5-5.0 and 6.8-7.0 respectively), or extracellular and cytosolic trehalases due of their localization in the cell (fig.3). The neutral trehalase, Nth1p, is responsible for hydrolysis of cytosolic trehalose. It has a pH optimum for activity at 7.0 and is responsible for the intracellular mobilization and/or recycling of trehalose (François & Parrou, 2001). Acid (or extracellular) trehalase, encoded by *ATH1*, is active as a monomer in yeast. It is highly specific for trehalose (*K*m ~ 0.8-5 mM), and has a high temperature optimum and high thermostability. Moreover, it does not appear to be regulated by a post-translational mechanism. Firstly, it was thought that Ath1p was only located in the vacuole but the protein also could be found in the cell surface, in the periplasmic space. However, the mechanism by which Ath1p is exported to the cell surface and the way in which it is retained still have to be elucidated (Parrou *et al.*, 2005).

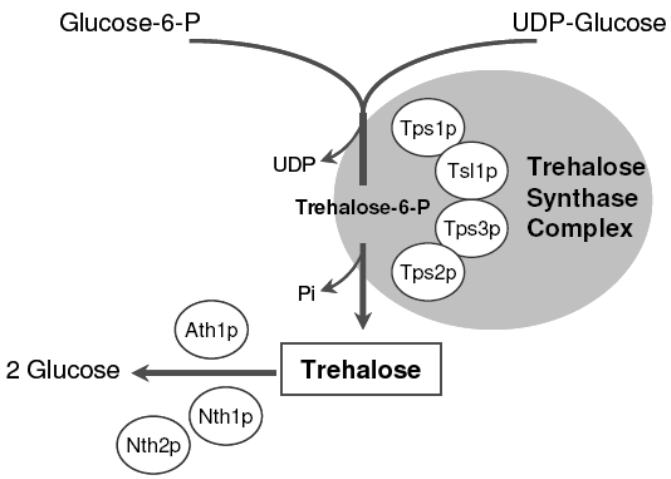

Fig. 3. Trehalose metabolic pathways in the yeast *Saccharomyces cerevisiae.*
Trehalose biosynthesis is catalysed by the trehalose synthase complex consisting of four subunits. The trehalose-6-phosphate synthase subunit (Tps1p) produces trehalose-6-P from UDP-glucose and glucose-6-P, which is dephosphorylated in trehalose by the trehalose-6-P phosphatase subunit (Tps2p). Tps3p and Tsl1p are two regulatory subunits that stabilize the complex. Trehalose is degraded by the neutral (Nth1p) or the acid (Ath1p) trehalases. The role of Nth2p in this degradation process has not yet been clarified. (Adapted from François and Parrou, 2001).

Trehalose assimilation

Trehalose can enter the cells by two different pathways. The most important one is associated with acid trehalase, and two alternatives have been proposed. (Nwaka *et al.*, 1996) proposed that trehalose reaches the vacuole by an endocytotic process in which it is degraded by the vacuolar acid trehalase. Jules *et al.* 2008 however, proposed a simpler explanation based on the finding that more than 90% of total acid trehalase in *S. cerevisiae* extracellular and cleaves extracellular trehalose into glucose in the periplasmic space (Jules *et al.*, 2008). A second pathway for trehalose assimilation couples trehalose transport by Agt1p with trehalose hydrolysis by Nth1p (Jiang *et al.*, 2000; Jules *et al.*, 2004; Jules *et al.*, 2008; Parrou *et al.*, 2005). This pathway will be referred to, later in Chapter 6.

Regulation of trehalose metabolism

Usually, a close correlation is observed between the trehalose content and the stress resistance of the cells (Wiemken, 1990). Genes related to trehalose metabolism have at least one stress response element (STRE) in their promoter, which is under the positive control of Msn2p/Msn4p (Estruch, 2000). However, the bare presence of STREs in the promoter does not explain the increase in trehalose level, and other factors must be considered. In fact, increases in the amount of enzymes, changes in enzymatic activities caused by allosteric effectors or covalent modification, and increases in the levels of substrates must be taken into account to explain the accumulation of trehalose in a specific stress condition (François & Parrou, 2001). Intracellular levels of trehalose are the result of a well-

controlled balance between enzymatic synthesis and degradation. Because the synthesis of 1 mol of trehalose from glucose requires 3 mol of ATP, but no ATP is produced when the disaccharide is hydrolysed by trehalase, a futile cycle will probably arise during simultaneous synthesis and degradation of trehalose (François *et al.*, 1991).

Saccharomyces cerevisiae as model for studies on stress tolerance

Yeast is a widely used organism both for industrial applications and pharmaceutical processes. Enlarged information about the regulation of its genes is the basis for further optimizations, like improvements via metabolic engineering. As a eukaryotic microorganism, it shares a significant part of its biological functions with higher organisms. Therefore, many experimental results derived from experiments with this easy-to-handle organism are also valid for mammals and even humans. *Saccharomyces cerevisiae* an important organism in both fundamental and applied research and has been one of the central model organisms for studies in genetics, biochemistry, cell biology, and more recently molecular biology and systems biology. In fact, we probably know more about the biology of the yeast cell than any other eukaryote. This makes *S. cerevisiae* an ideal model organism for studies on environmental-stress responses and stress tolerance. Such studies are of huge economic importance because of the numerous industrial applications of yeast, including brewing, winemaking, baking and, in more recent years, bioethanol production.

Torulaspora delbrueckii: an emergent yeast in baking industry

Nowadays the baker's yeast strains under use have been developed as a result of centuries of experience and selection resulting in a high degree of domestication best suited for bread making. Nevertheless, research to improve strains continues. Although methods of classical genetics (selection, mutation, and hybridization) are still very useful, novel methods such as protoplast fusion and genetic engineering have resulted in baker's yeast strains with even better technological properties (Hernandez-Lopez *et al.*, 2007; Randez-Gil *et al.*, 1999; Santos *et al.*, 2008). *S. cerevisiae* strains are generally used as baker's yeast, nevertheless the use of alternative species in bread making may allow to cope with the new and more demanding challenges in the baking industry. Dough leavening ability has been reported for yeast other than *S. cerevisiae*. *Issatchenkia orientalis*, *Pichia membranaefaciens*, and *Torulaspora delbrueckii* were the most abundant non-*Saccharomyces* species present in homemade corn and rye bread dough (Almeida & Pais, 1996b). Among them, the biotechnological interest in *T. delbrueckii* has increased in recent years due to its particularly high freezing and osmotic tolerance (Almeida & Pais, 1996a; Alves-Araújo *et al.*, 2004a; Hernandez-Lopez *et al.*, 2003). Thus, some *T. delbrueckii* strains are currently commercialized in Japan for regular (5% sucrose) frozen dough applications (Winde, 2003). In rural areas of Portugal bread is sometimes prepared with dough carried over from a previous making. Following an isolation program of yeasts from homemade corn and rye bread doughs, two strains of *T. delbrueckii*, PYCC 5321 and PYCC 5323 were selected

on the basis of combining simultaneously freeze tolerance and high leavening ability, characteristics desirable in baker's yeast. Leavening activity of *T. delbrueckii* PYCC 5321 and PYCC 5323 upon freeze-thaw appeared to be unaltered in comparison with the decrease of fermentative capacity in *S. cerevisiae* baker's yeast (Almeida & Pais, 1996a). This capacity could be explained, at least in part, by the slow rate of trehalose mobilization found in these strains and their ability to respond rapidly to osmotic stress. Indeed, the previous report of Almeida and Pais (1996b) showed that *T. delbrueckii* strains are not affected by fermentation before freezing. Furthermore, the superior freeze resistance of this yeast has been shown to relate to its higher capacity to preserve the integrity of the plasma membrane, associated to a lower increase of lipid peroxidation (Alves-Araújo *et al.*, 2004a). Thus, the application of these strains in bread making would have additional advantages, since a single strain can be used for most or all baking applications with the highest performance.

Although this yeast is widely commercialized in Japan the regular utilization of this yeast species in the bread-making industry has not been established due to some disadvantages. *T. delbrueckii* strains show considerable variation in their abilities to ferment and to assimilate carbon compounds, as galactose or maltose (Kurtzman, 1998), variable maltase activity and fermentative capacity (Hernandez-Lopez *et al.*, 2003). The small cell size is also an important disadvantage in the industrial dehydration process; filtration of cells for dehydration requires a long time, and even worse, filtration cannot be performed continuously because the filter for dehydration becomes clogged and must be changed frequently (Sasaki & Ohshima, 1987). Additionally, there is a lack of knowledge on the physiology and molecular biology of this yeast. Though the phylogenetic closeness of this yeast to *S. cerevisiae*, the differences observed between the two species, show that the behavior of *T. delbrueckii* cannot be directly inferred from that of *S. cerevisiae*. Therefore, in this work we centered our main attention on an investigation to gain insight into the physiology and biochemistry of *T. delbrueckii* sugar transport (see Chapter 3 and 5).

Following the work of Almeida and Pais (1996a) and to further exploit other collected doughs for the identification of additional strains of potential applied interested we needed an expedite method for strain differentiation. Since there were no published tools to differentiate *T. delbrueckii* isolates at the strain level a small study of the yeast flora present on the homemade corn and rye bread dough was carried out to test the suitability of two methods to distinguish strains of this species. Consequently, another topic studied in this work was the identification by molecular methods of *T. delbruecckii* strains in order to select for strains of potential value for the baking industry (Chapter 2).

An outlook on the classification of *Torulaspora delbrueckii*

The ascomycetous yeast genus *Torulaspora* is closely related to the genera *Saccharomyces* and *Zygosaccharomyces*. The history of *Torulaspora* reflects its close relationship with these two strains. The type strain of the genus was first described as *Saccharomyces delbrueckii* by Lindner in 1895. In 1904, the same author established the new genus *Torulaspora* and transferred *S. delbrueckii* to this genus as *Torulaspora delbrueckii* (van der Walt, 1970). In 1952 the genus *Torulaspora* was merged

into the genus *Saccharomyces* together with the genus *Zygosaccharomyces* by Lodder&Kreger van Rij (Lodder & Kreqer-Varni, 1952) and subsequently redefined in 1975 by van der Walt & Johanssen (van der Walt, 1975). In the third (1984) and fourth (1998) editions of 'The Yeasts: A Taxonomic Study,' *Torulaspora* accommodated three species: *T. delbrueckii, Torulaspora globosa* and *Torulaspora pretoriensis* (Kurtzman, 1998; Yarrow, 1984). In 2003 (Kurtzman, 2003), *T. delbrueckii, T. globosa, Torulaspora franciscae, Torulaspora microellipsoides* and *T. pretoriensis,* were assigned to the genus *Torulaspora.* More recently in 2008 (Limtong *et al.*, 2008) nine strains of a n*ew Torulaspora* species were isolated from natural samples collected in Japan and Thailand, which were named *Torulaspora maleeae sp. nov.*

Morphological and genetic characteristics of *Torulaspora delbrueckii*

The yeast *T. delbrueckii*, which is frequently found in alcoholic beverages, fruit juices, and high-sugar-containing foods (Esteve-Zarzoso *et al.* 2001) is often considered a spoilage yeast (Schuller *et al.* 2001) Most of the baking strains of *Torulaspora* display a vegetative growth as haploids (Kurtzman, 1998). It shows six chromosomes resolved by pulse-field gel electrophoresis in the range of 800 to1600 Kb (Oda & Tonomura, 1995) (see Chapter 2), a globular cell morphology, and smaller size than *S. cerevisiae* commercial baker's yeast.

Thesis outline

Two main trends are outlined here in this work. One relates to the molecular and physiological characterization of the osmo- and cryo-resistant yeast *T. delbrueckii* regarding sugar transport and metabolism. The other concerns the study of genes reported as cold-induced or essential for growth at freezing temperatures. Furthermore, we were also interested in select and characterize other *T. delbrueckii* strains with potential application in the baking industry. **Chapter 1** encloses a general introduction about *T. delbrueckii* traits that grants it potential application in baking industry. An outline on sugar metabolism and transport as well as freezing stress response is also introduced. As stated throughout this Chapter, in this thesis we will focus our attention on issues related with downstream applications of baker's yeast, in particular to sugar utilization patterns, respiro-fermentative metabolism (**Chapter 3**) and sugar transport (**Chapter 5)** in *Torulaspora delbrueckii,* and to cryoresistance (**Chapter 6**). Information concerning molecular techniques, as methods to distinguish between *T. delbrueckii* strains **(Chapter 2**), and an improved gene disruption method for this yeast (**Chapter 4**) will be also considered as it provides knowledge to further characterize this yeast. In **Chapter 7**, the results of this thesis are discussed in a broader perspective.

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Chapter 2

Molecular characterization of baker's yeast strains of *Torulaspora delbrueckii*

This chapter comprises parts from the following publication:

Pacheco A., Santos J., Almeida M. J. & Sousa, M. J. (2008). Molecular characterization and comparison of baker's yeast strains of *Torulaspora delbrueckii*. Submitted.

Abstract

Torulaspora delbrueckii PYCC 5321 and PYCC 5323 are two strains of industrial interest however, their variability has been neither fully investigated nor reported by molecular typing. To evaluate this variability mithocondrial DNA restriction analysis has been carried out for these *T. delbrueckii* strains. The analysis with *Hinf*I provided unique profiles for each strain that therefore can be considered as individual strains. These strains were also subjected to electrophoretic karyotyping and PCR amplification of ITS1-ITS4 region. Strain discrimination was obtained both by mtDNA restriction analysis and electrophoretic karyotyping. We propose that mtDNA restriction analysis is a convenient tool for quick identification of *T. delbrueckii* strains.

Introduction

Torulaspora species include some interesting strains that are of increasing relevance in the bread and wine industries. *Torulaspora delbrueckii* PYCC 5321 and PYCC 5323 (Portuguese Yeast Culture Collection,Caparica – Portugal) are of potential industrial interest in the bread-making industry (Almeida & Pais, 1996a; Almeida & Pais, 1996b; Hernandez-Lopez *et al.*, 2003; Hernandez-Lopez *et al.*, 2007). Indeed, these strains display high freeze/thaw tolerance (Almeida & Pais, 1996a; Alves-Araújo *et al.*, 2004a) and an exceptional resistance to osmotic and Na^+ injury (Hernandez-Lopez *et al.*, 2003; Ok & Hashinaga, 1997), making them suitable for application in the baking industry, particularly in frozen dough technology. Nevertheless, the regular use of *T. delbrueckii* species in the bread-making industry has not been established due, in some extent, to insufficient knowledge on this yeast. Nowadays, fermentation practices have included the use of selected yeasts in the form of active dry, fresh or frozen yeast. Therefore the accessibility of typing techniques that enable differentiation at the strain level is imperative for both baker's yeast users and producers, to assure that the commercialized yeast corresponds to the original selected strain. The characterization by the conventional methods require assessment of some 60–90 tests for a correct identification, which is laborious and time consuming (Deak & Beuchat, 1995*).* Also the reliability and reproducibility of these techniques is often questioned, because the evaluated characteristics can change according to growing conditions and in many cases they depend of the physiological state of the yeast (Golden *et al.*, 1994)*.* By contrast, methods using molecular biology analyze the genome independently of the physiological state of the cell. So the development of practical typing techniques that enable differentiation between *T. delbrueckii* strains is an essential tool for the implementation of this yeast in the baking industry. In this work we investigated the discriminatory power of mitochondrial DNA restriction analysis in the separation of *T. delbrueckii* strains, and used chromosome electrophoretic analysis to validate the results.

Materials and Methods

Yeast strains

T. delbrueckii PYCC 5321 and PYCC 5323 (Almeida & Pais, 1996a) were used throughout this work.

Mitochondrial DNA restriction patterns

For mitochondrial DNA restriction fragment analysis polymorphism (RFLP), yeast cells were cultivated in 5 ml YPD medium (24 h, 30 ºC, 160 r.p.m.) and DNA isolation was performed using a previously described method (Lopez *et al.*, 2001). Digestion was carried out with *Hinf*I restriction enzyme overnight at 37 ºC and prepared for a final volume of 20 µl as previously described (Schuller *et al.*, 2004).

Karyotyping analysis

Intact DNA for pulsed field gel electrophoresis (PFGE) was prepared in plugs as previously described (Ribeiro *et al.*, 2006). PFGE was run in a CHEF-DRII Chiller System (Bio-Rad, Hercules, CA). PFGE gels were run in 0.5% Tris borate-EDTA buffer at 12 °C with an angle of 120° with the following voltage and switch times: 480s → 900s, 3 v/cm for 10 hours; 240s → 480s, 3 v/cm for 15 hours; 120s → 240 s, 3 v/cm for 15 hours; 90 s, 6 v/cm for 10 hours and 60 s, 6 v/cm for 5 hours. Thereafter, gels were stained with 0.8% ethidium bromide solution for 45 min and distained for 20 min. Gels were visualized under UV light and analyzed using the EagleEye II Image Acquisition System (Stratagene, La Jolla, CA).

DNA manipulations

For general DNA manipulations see Appendix I.

Results and discussion

In order to check the suitability of two different genetic fingerprinting methods we carried out a small study of the yeast flora present on the home-made corn and rye bread dough in the northern area of Portugal to select autochthonous *T. delbrueckii* yeast strains. To reduce the number of strains for analysis we have previously screened 134 isolates by restriction pattern analysis of both PCR amplified 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS4 as described (Esteve-Zarzoso *et al.*, 1999), selecting only *T. delbrueckii* species (data not shown). The total length of ITS1-5.8S-ITS4 regions of 5.8S rRNA gene is identical for all *T. delbrueckii* strains (800 bp), hence this method cannot discriminate at strain level. From these results three isolates were selected for further studies. We applied a simplified (Schuller *et al.*, 2004) mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis and pulsed-field gel electrophoresis (PFGE) for discrimination between *T. delbrueckii* strains using PYCC 5321 and PYCC 5323 as comparative patterns.

Mitochondrial RFLP analysis has been widely applied to the characterization of reference and commercial *Saccharomyces cerevisiae* wine yeast strains (Esteve-Zarzoso *et al.*, 2004; Fernandéz-Espinar *et al.*, 2001; Guillamon *et al.*, 1996; Querol *et al.*, 1992; Schuller *et al.*, 2004; Schuller *et al.*, 2007) and strains belonging to other species (Guillamon *et al.*, 1997; Petersen *et al.*, 2001). Not all the enzymes used in this method reveal the same degree of polymorphism and it depends greatly on the species. Mitochondrial DNA RFLP analysis, using *Hinf*I, is associated with a high polymorphism and is a widely used genetic marker for the distinction of *S. cerevisiae* wine strains (Fernadéz-Espinar *et al.*, 2000; Lopez *et al.*, 2001; Querol *et al.*, 1992; Schuller *et al.*, 2007). RFLP of mitochondrial DNA using this enzyme, revealed two different profiles for *T. delbrueckii* PYCC 5321 and PYCC 5323, with slight variability. The major difference was found in the upper bands where the resolution is better (fig.1 arrows). Except for these two bands, the pattern of

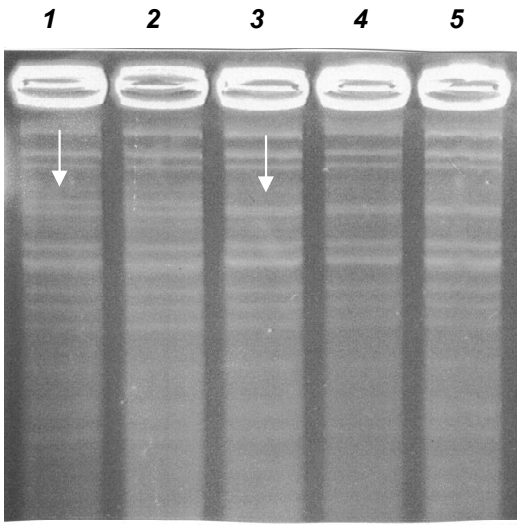

Fig. 1. mtDNA patterns obtained with the *Hinf*I restriction endonuclease.
Lane 1 – *T. delbrueckii* PYCC 5321 (profile I);
Lane 2 – isolate 1 (identical to profile I);
Lane 3 – *T. delbrueckii* PYCC 5323 (profile II);
Lane 4 – isolate 2 (identical to profile II);
Lane 5 – isolate 3 (identical to profile II).
Arrows indicate main differences between profiles I and II.

both strains is identical, indicating that these strains are genetically very closely related. Mitochondrial DNA RFLP for the other isolates resulted also in two different profiles (fig. 1), identical to ones of *T. delbrueckii* PYCC 5321 and PYCC 5323, demonstrating that these isolates probably correspond to the same strains. This is probably due to the fact that this study was performed in isolates from home-made doughs, that were collected in the same places from were *T. delbrueckii* PYCC 5321 and PYCC 5323 were first isolated. Additionally, it indicates that the features that make these strains suitable for the application in the baking industry, most probably give them adaptive potential in dough's environment, explaining why these strains were dominant among the *T. delbrueckii* isolates.

In order to confirm if the diversity found with mtDNA RFLP was also detectable in the karyotype of *T. delbrueckii* PYCC 5321 and PYCC 5323, the electrophoretic karyotypes of these two strains were ncompared. Karyotype analysis has been demonstrated to be a highly efficient technique to differentiate strains of *S. cerevisiae*, and was applied by numerous authors to characterize reference and commercial yeasts belonging to different species (Fernandéz-Espinar *et al.*, 2001; Petersen & Jespersen, 2004; Querol *et al.*, 1992; Schuller *et al.*, 2004; Schuller *et al.*, 2007). In this study PFGE revealed that the chromosomal DNA banding profiles of *T. delbrueckii* PYCC 5321 and PYCC 5323 differ markedly (fig. 2) ensuing that two different karyotypes could be defined on basis of size of putative chromosomes. Although under the conditions used, only four bands were clearly resolved, band intensities suggest that at least five to six chromosomes are present in both strains. These results are in, agreement with previous work were *T. delbrueckii* has been described to show six chromosomes (Oda & Tonomura, 1995). Also in accordance with the literature, the sizes of the putative chromosomes were very different between strains, *T. delbrueckii* PYCC 5323 having larger chromosomes than *T. delbrueckii* PYCC 5321. We demonstrate for the first time that *T. delbrueckii*

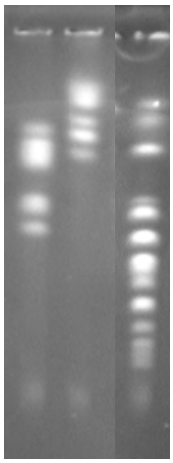

Fig. 2. Electrophoretic karyotype compariso between *T. delbrueckii* PYCC 5321 and PYCC 5323.
Lane 1 - *T. delbrueckii* PYCC 5321;
Lane 2 - *T. delbrueckii* PYCC 5323;
Lane 3 - *S. cerevisiae strain*.

PYCC 5321 and PYCC 5323 differed from each other clearly in the karyotyping profiles and RFLP of mitochondrial DNA. These data corroborate and complement that obtained in the past by the classical biochemical methodology (Almeida & Pais, 1996b), and represent an update to the understanding of *T. delbrueckii* populations present in bread dough's. Furthermore, the availability of functional typing tools that enable differentiation at the strain level is extremely important to the bread and wine industries, to assure traceability of the selected strains. RFLP of mitochondrial DNA is a simple technique and enables a great number of strains to be analyzed in less time. Furthermore, it is ideal for industry given its speed, safety and economy and also because it does not require sophisticated material or very specialized employees. We suggest the use of this reproducible and simple molecular method to routinely discriminate *T. delbrueckii* strains.

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Chapter 3

Sugar utilization patterns and respiro-fermentative metabolism in the baker's yeast *Torulaspora delbrueckii*

This chapter comprises parts from the following publication:

Alves-Araújo C., † Pacheco A., † Almeida M. J., Spencer-Martins I., Leão C. & Sousa M. J. (2007). Sugar utilization patterns and respiro-fermentative metabolism in the baker's yeast *Torulaspora delbrueckii*. *Microbiology* 153, 898-904.

†Both authors contributed equally to this work. **The thesis author contributed in particular with the determination of specific growth rates, biomass ans ethanol yields in media with sugars either alone or in mixtures. She also determined biomass yields at higher aeration and specific sugar consumption rates and maximum sugar transport capacity in single sugar media.**

Abstract

The highly osmo- and cryotolerant yeast species *Torulaspora delbrueckii* is an important case study among the *non- Saccharomyces* yeast species. The strain *T. delbrueckii PYCC 5321*, isolated from traditional corn and rye bread dough in northern Portugal, is considered particularly interesting for the baking industry. This paper reports the sugar utilization patterns of this strain, using media with glucose, maltose and sucrose, alone or in mixtures. Kinetics of growth, biomass and ethanol yields, fermentation and respiration rates, hydrolase activities and sugar uptake rates were used to infer the potential applied relevance of this yeast in comparison to a conventional baker's strain of *Saccharomyces cerevisiae*. The results showed that both maltase and maltose transport in *T. delbrueckii* were subject to glucose repression and maltose induction, whereas invertase was subject to glucose control but not dependent on sucrose induction. A comparative analysis of specific sugar consumption rates and transport capacities suggests that the transport step limits both glucose and maltose metabolism. Specific rates of CO_2 production and O_2 consumption showed a significantly higher contribution of respiration to the overall metabolism in *T. delbrueckii* than in *S. cerevisiae*. This was reflected in the biomass yields from batch cultures and could represent an asset for the large-scale production of the former species. This work contributes to a better understanding of the physiology of a non-conventional yeast species, with a view to the full exploitation of *T. delbrueckii* by the baking industry.

Introduction

Torulaspora delbrueckii and *Saccharomyces cerevisiae* are major constituents of the yeast flora present in corn and rye bread doughs (Hahn & Kawai, 1990; Almeida & Pais, 1996a). The biotechnological interest in *T. delbrueckii* has increased in recent years due to its particularly high freezing and osmotic tolerance (Almeida & Pais, 1996b; Ok & Hashinaga, 1997; Hernandez-Lopez *et al.*, 2003; Alves- Araújo *et al.*, 2004a). Moreover, strains of *T. delbrueckii* have been shown to display dough-raising capacities similar to those of commercial baker's yeasts (Almeida & Pais, 1996b), thus reinforcing their potential application in the baking industry. However, few reports exist on the genetics, biochemistry and physiology of *T. delbrueckii*, in contrast to the vast knowledge on the traditional baker's yeast *S. cerevisiae*. Two main aspects must be considered when selecting a yeast strain for the baking industry (Benitez *et al.*, 1996): effective biomass production in molasses, and dough-leavening ability. Sucrose is the primary carbon and energy source for growth in molasses, the industrial substrate used for large-scale baker's yeast production. Expression of invertase, the hydrolytic enzyme required to convert sucrose into glucose and fructose, is repressed by high glucose concentrations (Mormeneo & Sentandreu, 1982). Despite the high levels of invertase activity required for growth in molasses, there is evidence that the capacity of *S. cerevisiae* to ferment high sucrose concentrations, like those present in sweet bread doughs, is inversely related to the activity of this enzyme (Attfield & Kletsas, 2000). This is usually ascribed to the reduction in water activity resulting from sucrose hydrolysis and the consequent negative effect on yeast performance. Although there is a small amount of free sucrose and maltose, the maltose gradually released from starch as a result of amylolytic activity represents the major fermentable sugar in the dough (Ponte & Reed, 1982). Maltose metabolism requires the presence of both a maltose transporter and a maltase. In *S. cerevisiae*, the constitutive internal maltase is considered sufficient to hydrolyse maltose, and sugar utilization is limited by maltose uptake (Goldenthal *et al.*, 1987). The efficiency in gas production is determined by high maltase and maltose transport activities (Higgins *et al.*, 1999).

The specific growth rate is a key control parameter in the industrial production of baker's yeast (van Hoek *et al.*, 1998). The biomass productivity of *S. cerevisiae* is limited by the aerobic fermentation occurring in high-sugar media (Crabtree effect), demanding a high-oxygen fed-batch cultivation method to keep the sugar concentration low and avoid fermentative metabolism. Redirection of the respiro-fermentative flux at high sugar concentrations, and consequent improvements in biomass yields, have been successfully achieved by alleviating glucose repression, either by overexpressing a protein involved in the repressing pathway (Blom *et al.*, 2000) or by engineering glucose uptake rates (Otterstedt *et al.*, 2004). Notably, glucose transport has been shown by different authors to play a fundamental role in the fate of glycolytic flux in *S. cerevisiae* (Diderich *et al.*, 1999; Ye *et al.*, 1999).

In glucose-limited oxygen-sufficient chemostat cultures, *T. delbrueckii* shows biomass yields similar to those obtained for *S. cerevisiae* and consistent with fully respiratory growth. As the oxygen feed rate decreases, *S. cerevisiae* is the first to switch to a respiro-fermentative metabolism, already showing a decrease in biomass yield at oxygen tensions still able to sustain full respiration in *T.*

delbrueckii. However, *T. delbrueckii* shows considerably poorer growth than *S. cerevisiae* under strict anaerobic conditions (Visser *et al.*, 1990; Hanl *et al.*, 2005).

We have undertaken physiological and biochemical studies of *T. delbrueckii* in batch cultures with sugars present in molasses and in bread dough, using them alone and in mixtures. A strain isolated from traditional corn and rye bread dough in northern Portugal and showing particularly promising characteristics, *T. delbrueckii* PYCC 5321, was used. The resulting information on sugar utilization patterns, maltase and invertase activities, sugar uptake rates and respiration/fermentation rates contributes to a better evaluation of the potential offered by this yeast to the baking industry.

Materials and Methods

Microorganisms and growth conditions

The yeast strains used in this study were *Torulaspora delbrueckii* PYCC 5321, isolated from homemade corn and rye bread dough in northern Portugal, and *Saccharomyces cerevisiae* PYCC 5325, isolated from commercial compressed baker's yeast. They are both deposited at the Portuguese Yeast Culture Collection, Caparica, Portugal. Stock cultures were maintained at 4 ºC on slants of YPDA medium containing, per liter, 20 g glucose, 10 g peptone, 5 g yeast extract and 20 g agar. Since sucrose is the primary carbon and energy substrate present in beet or cane molasses used for industrial baker's yeast production, the inoculum for all experiments was prepared in YPS medium, containing, per liter, 20 g sucrose, 40 g peptone, 20 g yeast extract, 2 g KH_2PO_4 and 1 g $MgSO_4.7H_2O$. Peptone (0118-17) and yeast extract (0127-17) were from Difco and sucrose from Merck. Cells were harvested from a 24 h culture, washed and used as inoculum. Cultures were carried out in the same YP medium as used to prepare the inoculum but containing 20 g l^{-1} of sucrose, glucose, maltose or pairwise mixtures of these sugars. When indicated, yeasts were grown in a mineral medium (van Uden, 1967) supplemented with 20 g L^{-1} glucose, sucrose or maltose, with agitation in an orbital shaker (160 r.p.m.) at 30 ºC. Growth was followed by measuring the OD_{640} of the culture. At specified times during exponential growth, biomass dry weight was also determined.

Analytical procedures

To determine sugar and ethanol concentrations in the growth medium, the cultures were sampled and immediately centrifuged at 16 000 g for 3 min. The supernatant was frozen and kept at -20 ºC until analyzed. Quantitative analysis of sugar and ethanol was based on HPLC, using a Gilson chromatograph equipped with a 132-RI detector and a Hypersil-SS-100, H+ column at 30 ºC with a 5 mM H_2SO_4 solution as the mobile phase at a flow rate of 0.45 ml min^{-1}. Due to poor resolution of sucrose and maltose in this system, these two sugars when used in a mixture were determined enzymically using the sucrose/D-glucose UV colorimetric method and the enzyme α-glucosidase (Roche, 124036).

Biomass yields were determined from the slopes of plots of biomass dry weight versus consumed sugar during exponential growth. The ethanol yield was determined by dividing the maximum ethanol concentration obtained by the consumed sugar and expressed as g ethanol per g substrate carbon. Each specific sugar consumption rate (q_{sugar}) was determined by dividing the specific growth rate (μ) by the biomass yield (Y_x) during exponential growth on the respective sugar.

Enzyme assays

To obtain cell-free extracts for the determination of enzymic activities, 25–30 mg of cell mass (wet weight) was collected at different times during growth, sedimented by centrifugation, and washed twice with cold homogenization buffer (0.1 M potassium phosphate buffer, pH 6.5). The pellet was resuspended in 0.45 ml of the same buffer and transferred into a tube containing 0.5 ml acid-treated glass beads (0.5 mm diameter). The mixture was vortexed for four periods of 0.5 min, separated by 1 min intervals on ice, centrifuged for 5 min at 1000 g (4 ºC) and the supernatant used immediately for analysis. The total protein content in the cell-free extract was determined with the Bio-Rad protein assay kit based on the Bradford method (Bradford, 1976), and using bovine serum albumin as standard. Maltase activity was determined in the crude extract as described by Okada & Halvorson (1964) using *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) as substrate. One unit (U) is defined as the amount of enzyme that produces 1 µmol *p*-nitrophenol in 1 min under the assay conditions. Invertase activity was assayed as described by Niederacher & Entian (1987) and is expressed as µmol glucose released from sucrose in 1 min per mg protein (U mg $^{-1}$).

Maltose and glucose transport

For sugar transport assays, the cultures were sampled at the indicated times and cells were harvested by centrifugation, washed twice with ice-cold water, suspended in water to a density of 35–45 mg dry weight of cells ml^{-1} and kept on ice. Zero-trans influx of labeled maltose or glucose (Amersham) was determined at 30 ºC. Ten microliters of cell suspension was mixed with 30 ml 0.1 M potassium phosphate buffer (pH 5.0). The cell suspension was allowed to reach the temperature of the assay and the reaction started by adding 10 ml of an aqueous solution of [U-^{14}C] maltose (specific activity 610 mCi mmol^{-1}; 22.6 GBq mmol^{-1}) or [U-^{14}C] glucose (specific activity 310 mCi mmol^{-1}; 11.5 GBq mmol^{-1}) at the desired concentrations. After incubation for 5 s, 4.5 ml chilled water was added and the mixture immediately filtered through glass fiber filters (GF/C filters, Whatman). The cells on the filter were washed with 15 ml chilled water, the filter immersed in 5 ml scintillation liquid OptiPhase HiSafe II (LKB Scintillation Products) and the radioactivity measured using a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instrument Co.), with correction for disintegrations per minute. Non-specific binding of radiolabeled sugar to the yeast cells and filter was determined in parallel by pouring ice-cold water immediately before the addition of the labeled sugar. For each sugar concentration, the reaction was performed in triplicate.

Fermentation and respiration rates

Fermentation and respiration rates were determined using the standard Warburg method (Umbreit *et al.*, 1964). Yeast strains were grown on YP medium supplemented with 20 g l^{-1} glucose, sucrose or maltose. Cells were harvested at the exponential growth phase (OD_{640} 0.8–0.9), washed twice with water and suspended in cold water to a cell density 10-fold higher than the original culture. This suspension was diluted in 0.1 M KH2PO4 buffer, pH 5.0, to a cell concentration allowing measurements of CO_2 production and O_2 consumption in the manometer of the Warburg apparatus during a period of approximately 60 min. The experiments were started by the addition of the sugar solution (final concentration 20 g l^{-1}) to the cell suspension and performed at 30 ºC, in duplicate. Fermentation rates are expressed in mmol CO_2 produced per g dry weight of cells per hour, and respiration rates expressed in mmol O_2 consumed per g dry weight of cells per hour. The respiratory quotient (RQ) was calculated as the ratio between total CO_2 produced and the O_2 consumed.

Results

Growth and sugar utilization patterns

To characterize growth and sugar utilization patterns of *T. delbrueckii* PYCC 5321, the yeast was cultivated in YP medium with glucose, sucrose and maltose, either as single carbon and energy source or in mixtures. The curves in fig. 1 show the results obtained in sugar mixtures. In glucose-maltose (G-M) medium, maltose consumption became detectable only after glucose was no longer present (fig. 1a). No diauxic growth curve was observed, i.e. there was no lag period preceding the utilization of the second sugar. In glucose-sucrose (G-S) medium, the utilization of both sugars was simultaneous (fig. 1b). However, in S-M medium sucrose was apparently preferred to maltose (fig. 1c). Following an initial slow consumption of maltose, concomitant with sucrose utilization, a lag period was observed which lasted until sucrose had almost disappeared. Only then was maltose consumption resumed.

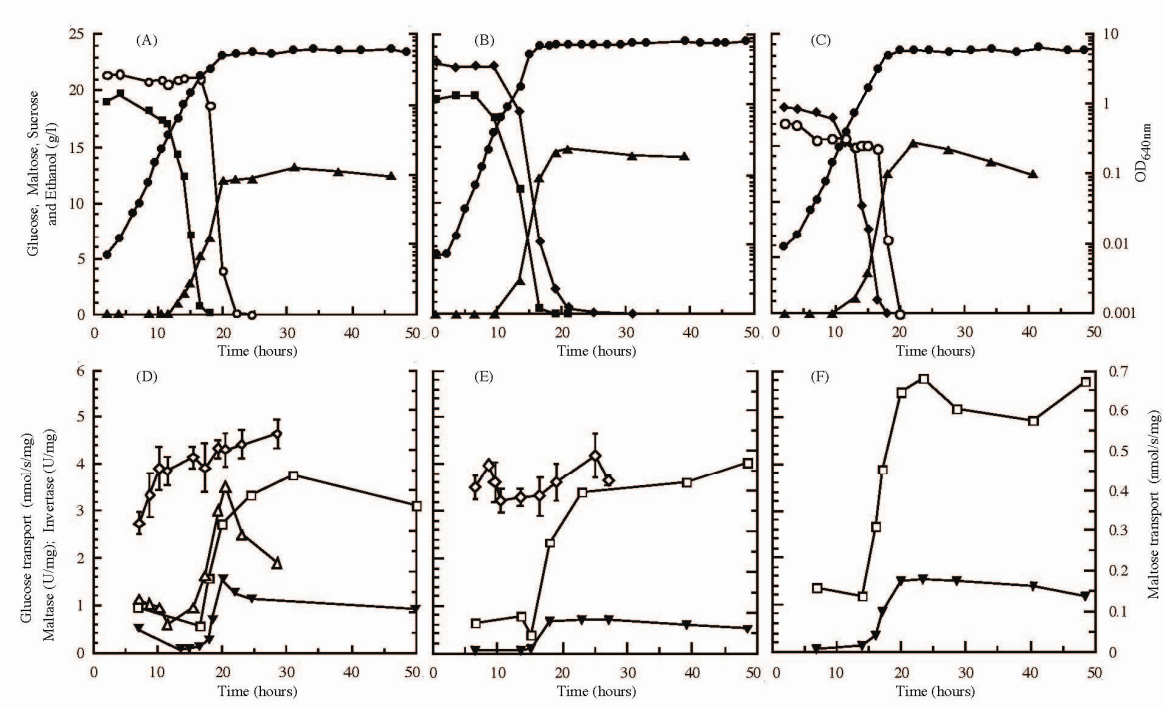

Fig. 1. Growth of *T. delbrueckii* in YP medium containing mixtures of glucose-maltose (a, d), glucose-sucrose (b, e) and sucrose-maltose (c, f).●, $OD_{640;}$■, glucose;○, maltose;◆, sucrose;▲, ethanol;▼, maltase activity [U (mg protein)$^{-1}$]; □, invertase activity [U (mg protein)$^{-1}$]; ◊, Δ, rate of glucose (◊) and maltose (Δ) transport [nmol s^{-1} (mg dry weight)$^{-1}$]. The data are representative of the results obtained in two independent experiments. The experimental variation was below 2% for all measurements except for glucose transport, where standard deviations are presented.

Specific growth rates, biomass and ethanol yields were estimated for the growth conditions tested and their values compared for *T. delbrueckii* PYCC 5321 and *S. cerevisiae* PYCC 5325 (table I). In single-sugar media, the growth rate values were similar, although slightly higher in glucose and sucrose media than in maltose medium for both yeasts. In mixed-sugar media, the values were similar to those obtained in single-sugar media (fig. 1, table I). As for biomass yields, typical values for fermentative metabolism were obtained in all cases. However, the biomass yields were slightly lower for *S. cerevisiae* than for *T. delbrueckii* (table I) in either glucose or maltose medium. Ethanol yields in sugar mixtures were higher when using *S. cerevisiae*, except in S-M medium, where similar values were observed (table I). For comparison, the pattern of maltose utilization when in the presence of glucose was also investigated for *S. cerevisiae* PYCC 5325. As expected, sequential sugar consumption was observed. However, maltose started to be consumed when glucose was still detectable in the medium (not shown), suggesting a higher glucose control over maltose metabolism in *T. delbrueckii* than in the commercial baker's yeast.

Table I - Specific growth rates, biomass and ethanol yields from batch cultures in YP medium supplemented with different sugars, either alone or in mixtures

		Carbon source					
		Glucose	**Sucrose**	**Maltose**	**Glucose-maltose**	**Glucose-sucrose**	**Sucrose-maltose**
T. delbrueckii PYCC 5321	μ	0.56±0.06	0.59±0.04	0.48±0.07	0.50±0.05	0.55±0.03	0.52±0.06
	Y_x	0.53±0.01	0.54±0.05	0.50±0.04	0.37±0.09	0.35±0.03	0.38±0.09
	Y_E	____	____	____	0.86±0.06	0.94±0.08	1.06±0.02
S. cerevisiae PYCC 5325	μ	0.67±0.05	0.68±0.04	0.62±0.05	0.65±0.06	0.64±0.04	0.64±0.07
	Y_x	0.48±0.10	0.54±0.10	0.42±0.04	0.31±0.03	0.39±0.05	0.33±0.06
	Y_E	____	____	____	1.08±0.05	1.10±0.10	1.07±0.06

μ, specific growth rate (h^{-1}); Y_x, biomass yield [g (g substrate carbon)$^{-1}$]; Y_E, ethanol yield [g (g substrate carbon)$^{-1}$]. Data are means ± SD of at least three independent experiments.

To further characterize the utilization of mixed sugars in *T. delbrueckii*, the activities of two key enzymes, invertase and maltase, were followed during the fermentations (fig. 1d–f). For all tested media, maltase and invertase activities remained at low levels while glucose (in G-M and G-S media) or sucrose (in S-M medium) were present and increased concomitantly as these sugars approached depletion. Interestingly, maximal maltase activity of *T. delbrueckii* in G-M medium was comparable to the value obtained with *S. cerevisiae*, although for the latter species maltase activity was detected before glucose was completely consumed (results not shown), a result in accordance with the observed maltose utilization when glucose was still being consumed. Furthermore, while maltase activity in *T. delbrueckii* reached higher values whenever maltose was present in the medium, maximal invertase activity in the absence of sucrose (G-M medium) was similar to that found in glucose-sucrose medium (fig. 1d–f). However, both enzymes were subject to glucose repression. Similar results were observed upon growth in synthetic medium (data not shown), hence excluding the possible interference of residual amounts of sugars contained in YP-based media.

Sugar transport

To investigate a possible relation between specific sugar consumption rates inferred from the values shown in table I ($q_{sugar}=\mu/Y_x$) and the first step of maltose and glucose metabolism, the transport of these two sugars was evaluated during the fermentations in mixed-sugar media (fig. 1d, e). Just like *S. cerevisiae, T. delbrueckii* is known to transport maltose through a maltose-H^+ symport mechanism, inducible and subject to glucose repression (Alves-Araújo *et al.*, 2004b). Accordingly, in G-M medium we observed that maltose transport capacity increased only after glucose exhaustion (fig. 1d). The maximum maltose transport capacity obtained [0.41 nmol s^{-1} (mg dry weight)$^{-1}$: fig. 1d)] was about one and a half times lower than that obtained for cells grown in YP maltose medium [V_{max}=0.66 nmol s^{-1} (mg dry weight)$^{-1}$], which in turn is lower than the estimated specific maltose consumption rate

[*q*maltose=1.8±0.3 nmol s^{-1} (mg dry weight)$^{-1}$]. This suggests that maltose uptake may be limiting maltose metabolism. A similar analysis was conducted for glucose. Glucose transport in *T. delbrueckii* follows a biphasic Michaelis–Menten kinetics with low- and high-affinity components (Alves-Araújo *et al.*, 2005). During exponential growth in G-M and G-S media, glucose uptake rates were very similar (fig. 1d, e). The estimated specific glucose consumption rate [*q*glucose=4.1±0.4 nmol s^{-1} (mg dry weight)$^{-1}$] in YP glucose medium was comparable to the total capacity of glucose transport [V_{max}=3.96±0.56 nmol s^{-1} (mg dry weight)$^{-1}$ in G-M and 3.60±0.32 nmol s^{-1} (mg dry weight)$^{-}$1 in G-S], indicating that glucose metabolism may well be limited by glucose transport.

Sugar metabolism

Respiratory and fermentative capacities of *T. delbrueckii* PYCC 5321 and *S. cerevisiae* PYCC 5325 cells grown in YP with glucose, maltose or sucrose as the only carbon and energy sources were determined using the Warburg method. The results, expressed as specific CO_2 production (qCO_2) and oxygen consumption (qO_2) rates, are presented in table II. The data obtained with *T. delbrueckii* show that all sugars tested are essentially fermented (77–88% of the total sugar supplied) and that the fermentation rates were higher for sucrose and glucose than for maltose (table II), which is in accordance with the lower values obtained for *q*maltose and *μ*maltose (table I). It is noteworthy that the qCO_2 values for sucrose and glucose were always similar, irrespective of the sugar used for growth (table II). However, fermentation rates obtained with maltose were significantly higher ($P<0.001$) in maltose-grown cells than in either sucrose or glucose-grown cells (table II).

Table II -Specific fermentation and respiration rates of *T. delbrueckii* and *S. cerevisiae* grown in media with different sugars. Yeasts were grown in YP medium supplemented with 20 g l^{-1} of the indicated sugar, and harvested at the exponential phase (OD_{640} 0.8–0.9). CO_2 production and O_2 consumption rates are expressed as mmol h^{-1} (g dry weight)$^{-1}$. RQ=qCO_2 (total)/qO_2. Values are means±SD of three independent experiments.

Sugar in medium	Sugar in assays	*T. delbrueckii* PYCC 5321			*S. cerevisiae* PYCC 5325		
		qCO_2	qO_2	RQ	qCO_2	qO_2	RQ
Glucose	glucose	6.36±0.43	2.92±0.70	3.18±0.79	14.26±0.71	1.64±0.44	9.70±2.6
	sucrose	6.42±0.85	___	___	14.22±0.52	___	___
	maltose	2.79±0.22	___	___	11.54±1.17	___	___
Sucrose	glucose	5.57±1.26	___	___	12.97±0.63	___	___
	sucrose	6.94±0.54	2.86±0.26	3.43±0.41	12.29±0.81	2.79±0.19	5.41±0.51
	maltose	2.74±0.09	___	___	10.08±1.05	___	___
Maltose	glucose	5.65±0.92	___	___	10.13±0.44	___	___
	sucrose	6.07±0.91	___	___	10.50±1.26	___	___
	maltose	5.59±0.50	5.01±0.61	2.12±0.32	12.45±1.20	2.77±0.12	5.50±0.58

S. cerevisiae PYCC 5325 displayed a somewhat different behaviour. The fraction of glucose fermented (approx. 96–97 %) was higher than in the case of sucrose or maltose (around 93 %), as readily inferred from the RQ values (table II). No significant differences between the fermentation rates of maltose were observed in cells grown with any of the three sugars (table II), in contrast to the values obtained for glucose and sucrose fermentation. In both cases, the qCO_2 values were found to be higher in glucose or sucrose-grown cells and lower in maltose-grown cells ($P<0.001$ and $P<0.01$, respectively).

A comparative analysis between *T. delbrueckii* and *S. cerevisiae* regarding the specific oxygen consumption rates (qO_2) estimated with the different sugars showed similar values for sucrose, whereas the values almost doubled for *T. delbrueckii* in the case of glucose and maltose (table II). As stressed above, the relative contribution of respiration to sugar catabolism is always higher in *T. delbrueckii*, the RQs varying between 3.43 (sucrose) and 2.12 (maltose). For *S. cerevisiae*, RQ values were in the range 5.41–9.70, which reflects the higher fermentative capacity of this yeast.

Discussion

T. delbrueckii is nowadays an important case study among the non-*Saccharomyces* yeast species, with particular relevance to the baking and wine industries. Among the most important

characteristics of a good baker's yeast is the dough-leavening ability, which implies the efficient fermentation of both maltose and glucose, and high biomass productivities on sucrose, the major sugar in molasses used as raw material to produce the yeast. Although the patterns of sugar utilization by *T. delbrueckii* are very similar to those described for *S. cerevisiae* (Mormeneo & Sentandreu, 1982; Needleman, 1991; Gancedo, 1998), a few significant differences were observed. In G-M medium the pattern was almost identical, the increase in maltose transport and maltase activities clearly coinciding with the outset of maltose consumption. Hence, there is an apparent coregulation of these proteins, both being subject to glucose repression and induction by maltose. The results obtained are consistent with the previous identification of a bifunctional MAL promoter in *T. delbrueckii* PYCC 5321, shared by maltase and maltose transporter genes, including Mig1p and UASMAL consensus binding sites (Alves-Araújo *et al.*, 2004b). The glucose control over maltose metabolism was stricter in *T. delbrueckii* PYCC 5321, since *S. cerevisiae* PYCC 5325 started to consume maltose when glucose was still detectable in the medium. In glucose-maltose mixtures, under laboratory culture conditions, this differential behaviour of the two species could lead to an undesirable delay in CO_2 production from maltose by *T. delbrueckii*. However, this advantage exhibited by *S. cerevisiae* is counteracted under the conditions prevailing in bread dough by the higher osmotolerance of *T. delbrueckii*. Indeed, the gas production capacity of *T. delbrueckii* PYCC 5321 in lean dough was slightly lower than the leavening capacity of *S. cerevisiae* PYCC 5325 (Almeida & Pais, 1996b) and slightly higher than the rates obtained with other commercial baker's yeast strains (Hernandez-Lopez *et al.*, 2003). The differences reported by the latter authors were even more pronounced in sweet, sucrose-added, doughs. In sucrose medium, *T. delbrueckii* PYCC 5321 showed a lower growth rate than *S. cerevisiae* PYCC 5325, although the biomass yields were equivalent. This is consistent with the higher contribution of respiration to the overall sugar metabolism in *T. delbrueckii*. Since the biomass productivity, in industrial fed-batch cultures, is limited not only by the substrate concentration but also by the oxygen available, the growth potential of *S. cerevisiae* is countered by its requirement for a more careful monitoring of the oxygen tension, to prevent ethanol production. The level of invertase activity in rich YP medium is similar for both yeasts and the regulatory mechanisms for this enzyme appear to be the same. A correlation between invertase activity and sucrose consumption is unclear and needs further investigation.

Overall, the experimental evidence points to sugar transfer rates into the cell limiting the efficiency of the fermentation. In the case of sugar mixtures with maltose, the inhibitory effect of maltose on glucose uptake, which is known to occur in *S. cerevisiae* (Diderich *et al.*, 1999) and was also found in *T. delbrueckii* (Alves-Araújo *et al.*, 2005), could reinforce this limitation. In particular, in S-M mixtures (fig. 1c) the maltose concentration surpasses by far the glucose resulting from extracellular sucrose hydrolysis, thus possibly hindering glucose (and fructose) utilization. This would mean that, in the absence of maltose, sucrose could be consumed faster, which in reality was not observed. On the contrary, it seems that the glucose being released from sucrose through the action of the invertase inhibits maltose metabolism through mechanisms of glucose repression. More detailed studies are required to evaluate these aspects. Despite the clear fermentative metabolism of *T. delbrueckii*, with the production of high ethanol yields in batch cultures with each of the sugars tested, our data on the specific rates of CO_2 production and O_2 consumption, estimated with the Warburg manometric

technique, showed a higher contribution of respiration in *T. delbrueckii* compared to *S. cerevisiae*. It is worth noting that during batch cultivation the available oxygen rapidly reaches limiting concentrations, thereby favouring fermentative metabolism. In fact, when biomass yields were determined in YP medium, with either glucose, sucrose or maltose, using higher aeration rates a very significant increase in biomass yields (from 20 %, in glucose or sucrose medium, to 80 %, in maltose medium) was observed (not shown). As emphasized above, a more efficient modulation of the respiratory metabolism in *T. delbrueckii* under aerobic conditions represents an asset for the large-scale production of yeast.

As a final remark, the strain *T. delbrueckii* PYCC 5321 used in the present work was reported to display a much higher freezing and osmotic tolerance than *S. cerevisiae* (Almeida & Pais, 1996a, b; Alves-Araújo *et al.*, 2004a), properties of special interest for the baking industry. In addition to these characteristics, our results show that *T. delbruecki* behaves very similarly to *S. cerevisiae* with respect to sugar utilization and regulation patterns. This work also indicated that maltose uptake is a good target for metabolic engineering and improvement of *T. delbrueckii's* performance in bread doughs. The present study further contributes to the characterization of *T. delbrueckii* PYCC 5321 at the physiological and biochemical levels, bridging a gap for its exploitation by the baking industry and increasing knowledge on the so-called non-conventional yeast species.

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Chapter 4

Improved gene disruption method for *Torulaspora delbrueckii*

This chapter comprises parts from the following publication:

Pacheco A., Almeida M J & Sousa M J. 2008. Improved gene disruption method for *Torulaspora delbrueckii*. FEMS Yeast Research (published online).

Abstract

PCR-based disruption cassettes are one of the most commonly used strategies for gene targeting in *Saccharomyces cerevisiae*. The efficiencies of gene disruption using this conventional method are highly variable among species, and often quite low with non-conventional yeasts. Here we describe an improved strategy to obtain deletion mutants in baker's yeast *Torulaspora delbrueckii,* one of the most abundant non-*Saccharomyces* species, present in home-made corn and rye bread dough.

Introduction

Torulaspora delbrueckii, one of the yeast species most frequently found in home-made corn and rye bread dough (Almeida & Pais, 1996b), has been recognized as the most promising alternative to industrial *Saccharomyce cerevisiae* baker's yeast strains (Almeida & Pais, 1996a). In the wine industry, interest in *T. delbrueckii* has also increased in recent years due to its positive contribution to aroma development (Ciani & Maccarelli, 1998). Nevertheless, there is a lack of knowledge on the physiology, biochemistry and molecular biology of this organism and an in-depth investigation is required. For this, construction and analyses of mutant strains in *T. delbrueckii* are of utmost importance; however, the genetic tools available are very scarce. Typically in yeast the disruption of a gene is accomplished by transforming cells with a gene-targeting fragment (cassette) containing a selectable marker, such as a gene conferring drug resistance or nutrient autotrophy, flanked by upstream and downstream sequences of the gene of interest (Rothstein, 1991).

Usually, these cassettes are generated by PCR, using primers composed of both bordering regions of the target gene and part of a selectable marker gene and subsequently used to transformed yeast cells through a transformation protocol, usually the lithium acetate TRAFO method (Schiestl & Gietz, 1989) (fig.1).

In fact, the demonstration that only very short sequences of yeast DNA on either side of a marker gene were needed for efficient integration into the *S. cerevisiae* genome by homologous recombination (Manivasakam *et al.*, 1995) allowed the generalized use of gene disruption cassettes generated by PCR (fig.1) in yeast (Dujon, 1998; Winzeler *et al.*, 1999). However, a frequently occurred problem with non-conventional yeast is that standard recognized methods for *S. cerevisiae* not always work with other yeast or even with non -laboratorial *S. cerevisiae* strains. We were confronted with that problem when we attempted to disrupt *LGT1* gene in *T. delbrueckii.* Using the conventional method of a PCR-amplified disruption cassette with a short flanking homology (SFH-PCR) (Wach *et al.*, 1994), we were unable to obtain Δ*lgt1* mutants.

This work describes a strategy that can be a useful alternative to the conventional PCR-based gene disruption for the yeast *T. delbrueckii*. Furthermore, this method may also be applied to other non-conventional yeasts, where correct gene disruption with the commonly used short flanking homology cassettes is frequently very low.

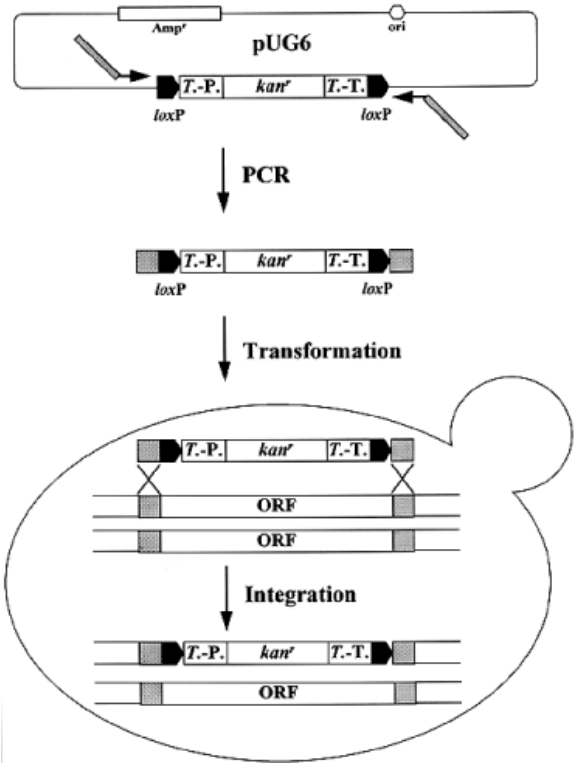

Fig. 1. Conventional method of PCR-based disruption cassette. Gene disruption experiment using two oligonucleotides that carry at their 3'-end a segment (arrow) homologous to sequences left and right of the *loxP* - *kanMX* - *loxP* module on plasmid pUG6 and at their 5'-end a segment (shaded box) homologous to the ORF to be disrupted. Plasmid pUG6 is used as PCR template to generate the disruption cassette. Adapted from (Guldener *et al.*, 1996).

Materials and methods

Strains, Media, and Growth Conditions

Torulaspora delbrueckii PYCC 5321 was used throughout this work. Yeast cells were cultured at 30 ºC in YPD media (1% yeast extract, 2% peptone and 2% glucose). Yeast transformants containing the geneticin resistance module (kanMX4) were selected on YPD-agar plates supplemented with 300 µg L^{-1} of G418 (geneticin), added after autoclaving and cooling to 60 ºC (G418 was used from several suppliers - all work well). LB medium were prepared as previously described (Sambrook J, 1989). When necessary 100 µg ml^{-1} of ampicillin was added to standard LB plates or liquid after autoclaving and cooling to 60 °C. A sterile filtered of the antibiotic stock solution was used in both cases.

Reagents

Oligonucleotides (0.05 µmol scale) were purchased from MWG Biotech, Germany and are described in table I. Restriction and modification enzymes were from Roche Applied Science, Germany. Accuzyme DNA Polymerase was obtained from Bioline, Germany.

Table I. Oligonucleotides used in this study

Sequence 5‘ to 3’	**Comments**
TCCGTGAATGTTCACCCAGTT	Disruption cassette construction
TGGTCTCCACTTCTTCCAAG	Disruption cassette construction
GGGTTCCACCAGACAGAAGAGGTG	Verify correct targeting of the kanMX4 module
CGTGATATCCTCCATTTCACA	Verify correct targeting of the kanMX4 module

Construction of the *Torulaspora delbrueckii* LGT1 disruption cassette

*Pvu*II-*loxP-KanMX-loxP-Spe*I fragment released by restriction from plasmid pUG6 (Goldstein & McCusker, 1999) was cloned into *Bal*I/*Nhe*I- restricted YepHxt6 (Alves-Araújo *et al.*, 2005) (a plasmid containing *LGT1* ORF and part of the gene promoter and terminator regions), creating YLGT1kan. The TdLGT1 disruption cassette containing the *loxP-KanMX-loxP* module (Guldener *et al.*, 1996), flanked by 320 bp and 345 bp (5′ and 3′ sides, respectively) homologous to each margin of *LGT1*, was generated by PCR using the YLGT1kan plasmid as template (fig.1). DNA cloning and manipulation were performed according to the standard protocols as described (Sambrook J, 1989). For general DNA manipulations see Appendix I.

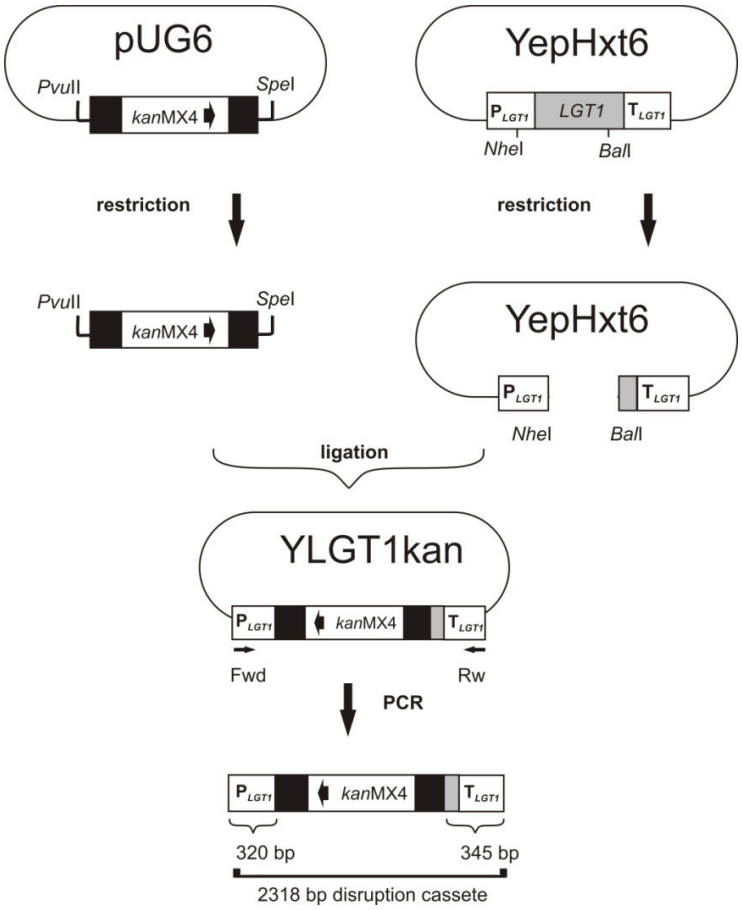

Fig. 1. Schematic illustration of the construction of *Torulaspora delbrueckii LGT1* disruption cassete. pUG6 plasmid was digested with *Pvu*II and *Spe*I to release de KanMX4 module, which confers resistance to geneticin (left side of the scheme). In parallel YepHxt6 plasmid (which contains *LGT1* ORF and part of the gene promoter and terminator regions) was restricted with *Nhe*I and *Bal*I, removing nearly the entire ORF (right side of the scheme). Afterward the *Pvu*II-loxP-KanMX-loxP-*Spe*I released from pUG6 was cloned into *Bal*I/*Nhe*I restricted YepHxt6, creating YLGT1kan plasmid, the template to generate the *LGT1* disruption cassette. Using specific primers to *LGT1* promoter and terminator regions, the disruption cassette (2318 bp) containing the marker module flanked by 320 and 345 bp (5′ and 3′ sides, respectively) *LGT1* homologous regions was generated by PCR. This cassette was used to transform *T. delbrueckii* with a modified LiAc transformation protocol (described in the text). P_{LGT1} and T_{LGT1} are the promoter and terminator regions, respectively, of *T. delbrueckii LGT1* gene. Restriction enzyme sites and the sizes of the DNA fragments are shown. Arrows at either end of the module represent the oligonucleotides used for PCR (table I).

Yeast transformation

Torulaspora delbrueckii cells were grown overnight in complete medium to stationary phase. Ten milliliters of this culture was diluted into fresh 2x YPD medium to an optical density at 600 nm of 0.25, and grown for an additional 4 hours (at least two duplications). Cells were collected, washed first with 25 ml of cold water, and then with 5 ml of 0.1 M LiAc /1xTE and next resuspended in 200 µl of this last solution. Fifty microliters of the cell suspension was pelleted and the supernatant was removed. Transformation mixtures contained the pelleted cells and 240 µl of polyethyleneglycol/ LiAc-sol (50%

polyethyleneglycol 4000 in LiAc-sol), 36 µl of 1M LiAc /1xTE, 30 µl of DNA carrier (10 mg/ml- boiled for 10 min, and 5 min on ice), >5 µg of the disruption cassete, 25 µl of DTT 1 M and 19 µl of H_20. This mixture was briefly vortexed and subsequently incubated for at least 30 min at 30 ºC, followed by a heat shock of 20 min at 42 ºC. Cells were then pelleted and resuspended in 2x YPD supplement with 100 µg mL^{-1} geneticin and incubated overnight (instead of the usual 4-hour recovery time). Finally, cells were plated onto selective plates of YPD supplemented with 300 µg mL^{-1} and incubated for up to 4 days.

Results and Discussion

LGT1 was the first gene identified as coding for a hexose transporter in *Torulaspora delbrueckii* but evidences indicate the presence of other hexose transporters in this yeast (Alves-Araújo *et al.*, 2005). For this reason, we could not screen for TdLGT1 disruptants by searching for a clear-cut phenotype, because loss of *LGT1* might be compensated by the activities of other genes and is not expected to impair glucose growth capacity. Several disruption cassettes containing the geneticin-resistance module flanked by 40 bp sequences homologous to different *LGT1* bordering regions were constructed and numerous transformants of several disruption rounds were obtained. However, in our experiments all the tested transformants were geneticin-resistant due to misintegration of the TdLGT1 disruption cassette and few to spontaneous resistance. Indication of a low efficiency of homologous recombination in this yeast, has also emerged before in the attempt to disrupt the *TdMAL11* gene (Alves-Araújo *et al.*, 2004b); still, in that case, a phenotypic analysis was possible because *TdMAL11* null mutants were unable to grow on maltose medium.

To improve homologous recombination frequency in *T. delbrueckii*, we first developed a new strategy to obtain a TdLGT1-targeting cassette harboring longer arms. Our approach was to insert a marker-resistance module into the core of *LGT1* gene, and then using this construction as a template, generate, by PCR amplification, a TdLGT1 disruption cassette with longer flanking regions.

Afterward in an attempt to further optimize the yield of *LGT1* disruption, some individual parameters of the lithium acetate TRAFO method described by (Schiestl & Gietz, 1989) were tested for their contribution to the transformation efficiency.

On the whole strategy two important modifications turned out to be particularly relevant: the size of the disruption cassette and the incubation (recovery) period of the cells during the transformation protocol. Efficiency using the TdLGT1 cassette together with the modified transformation protocol was extremely high when compared with the conventional method (none, using the conventional method and 12/16 using the improved method). Therefore, this method proved to be a useful alternative to the conventional PCR-based gene disruption for the yeast *T. delbrueckii*. Moreover, the strategy described here could also be applied to other non-conventional yeasts, where correct gene disruption with the commonly used short flanking homology cassettes is frequently very low.

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Chapter 5

A new hexose transporter from *Torulaspora delbrueckii*

This chapter comprises parts from the following publication:

Pacheco A., Hernandez-Lopez MJ., Almeida MJ., Prieto JA., Randez-Gil F. and Sousa MJ. (2008). A new hexose transporter from *Torulaspora delbrueckii*. Submitted manuscript.

Abstract

Most of yeast biotechnological applications rely on their ability to efficiently ferment a great variety of sugars. This property is closely related to their sugar transport capacity, which has been widely considered a rate-limiting step of sugar metabolism. In a previous work, we have cloned and characterized a glucose transporter gene, *LGT1,* from *Torulaspora delbrueckii*, a yeast specie receiving increasing interest both from the baking and wine industries. Here we show that disruption of this gene leads to a significant, although not severe, decrease in glucose transport in comparison with the wild-type strain. Southern blot analysis of the yeast genome, using a fragment homologous to *LGT1* as a probe, showed the presence of several genes with high homologous sequences. The occurrence of several hexose transporters had also been suggested by the isolation, from a genomic library of this strain, of several plasmids that could complement the glucose growth defect of a hexose transport-null strain of *Saccharomyces cerevisiae*. DNA sequencing of the insert from one of the recovered plasmids, revealed the presence of a 1673 bp-length uninterrupted open reading frame (ORF). This ORF, named *IGT1*, is located upstream of *LGT1* and displays a high homology to this gene and to other yeast glucose transporter genes. Functional characterization of Igt1p in *S. cerevisiae hxt*-null strain revealed that it encodes an intermediate-affinity transporter able to mediate the uptake of glucose, fructose and mannose. Furthermore, similarly to *S. cerevisiae* Hxt2p, apparent K_m of Igt1 transporter could be modulated by medium glucose concentration. Cells of *S. cerevisiae hxt*-null strain transformed with *IGT1,* when grown in 0.1% glucose displayed biphasic uptake kinetics with an intermediate- (K_m = 6.5± 2.0 mM) and a high-affinity (K_m= 0.10± 0.01 mM) component. These evidences suggest that similarly to that described for other yeasts, *T. delbrueckii* contains several hexose transporters, which display different and complementary affinities for glucose.

Introduction

Torulaspora delbrueckii is a non-conventional yeast strongly related to *Saccharomyces. cerevisiae* (James *et al.*, 1996; Oda *et al.*, 1997) and of increasing industrial interest. It has a positive effect on the taste and aroma of alcoholic beverages (Ciani & Maccarelli, 1998; Ciani, 1995) and exhibits low production of acetaldehyde, acetoin, acetate, and ethyl acetate during grape must fermentation (Cabrera, 1988; Ciani & Ferraro, 1998; Martinez, 1990). Also the use of *T. delbrueckii* under standard conditions, in mixed or sequential culture with *S. cerevisiae*, has been proposed as a way of reducing the acetic acid content in wine (Ciani *et al.*, 2006; Ciani, 1995). Some *T. delbrueckii* strains are also frequently found in isolates from traditional corn and rye bread doughs from northern Portugal (Almeida & Pais, 1996b). These strains exhibit a very good baking ability and an exceptional resistance to osmotic (Hernandez-Lopez *et al.*, 2003) and freeze-thaw stresses (Almeida & Pais, 1996a), opening the possibility of their application in high-sugar and frozen-dough fermentations. These features made this organism a *candida*te of potential value for the baking industry.

The transport of hexoses across the plasma membrane in *S. cerevisiae* is known to be a critical step in the metabolism of carbon compounds during fermentation (Diderich *et al.*, 1999a; Ye *et al.*, 1999), and we have previously shown that the same happens with *T. delbrueckii* (Alves-Araújo *et al.*, 2007). Glucose uptake has been most broadly studied in *S. cerevisiae* (Boles & Hollenberg, 1997), and it was formerly described in a quite simplified approach by means of a high-affinity and a low-affinity transport system (Bisson & Fraenkel, 1983; Coons *et al.*, 1995). The sequencing of the yeast genome and subsequent characterization of mutant strains have altered this view unrevealing a large number of hexose transporters. Microorganisms use a variety of different membrane-bound transport proteins of the Major Facilitator Superfamily (MSF), a group which appears to share a common ancestral origin (Andre, 1995). The number of hexose transporters among yeast is very variable, with 20 hexose transporters in *Candida albicans,* seven in *Kluyveromyces lactis,* six in *Schizosaccharomyces pombe* and three in *Pichia stipitis*. In *Saccharomyces. pastorianus* and in *Zygosaccharomyces bailii* until now just one hexose transporter has been described. In *S. cerevisiae* hexoses are transported by facilitated diffusion, and this yeast has more than 20 different hexose transporters (Hxt), a family of 18 individual systems (Hxt1-17 and Gal2) and two related signal proteins (Snf3p and Rgt2p). Although most Hxt homologous proteins have been shown to be able to transport hexoses, not all are functional transporters and these genes are likely pseudogenes (Wieczorke *et al.*, 1999). In a null strain lacking all known hexose transporters, glucose consumption and transport activity are completely abolished (Wieczorke *et al.*, 1999). Other studies revealed that transporters Hxt1 to Hxt4 plus Hxt6 and Hxt7 are the most important for the uptake of glucose (Boles & Hollenberg, 1997; Diderich *et al.*, 2001; Ozcan & Johnston, 1999; Wieczorke *et al.*, 1999). *T. delbrueckii* PYCC 5321 display a mediated glucose transport activity best fitted assuming a biphasic Michaelis–Menten kinetics with a low- and a high-affinity component. Until now, just one glucose transporter has been identified in *T. delbrueckii*, the low-affinity glucose transporter *LGT1* (Alves-Araújo *et al.*, 2005). Functional characterization of the *LGT1* gene product in *S. cerevisiae* revealed that it encodes a low-affinity transporter, able to mediate the uptake of glucose and fructose

(Alves-Araújo *et al.*, 2005). Here we show the existence of several genes with high homology to *LGT1*, revealed by Southern blot analysis of this yeast genome. The occurrence of several hexose transporters had previously been suggested by the isolation, from a genomic library of this strain, of several plasmids that could complement the glucose growth defect of the *S. cerevisiae* hexose transport-null mutant (Alves-Araújo *et al.*, 2005). Consequently we report, the isolation and characterization of a second glucose transporter *IGT1* (the first of intermediate affinity), in the non-conventional yeast *T. delbrueckii*. A kinetic analysis of the sugar transport driven by *Igt1*p in a hexose transport-null mutant strain of *S. cerevisiae* is also described.

Materials and Methods

Strains, Media, and Growth Conditions

Torulaspora delbrueckii PYCC 5321, and a glucose transport-null mutant of *Saccharomyces cerevisiae*, EBY.VW4000 (Wieczorke *et al.*, 1999) were used throughout this work. Yeast cells were cultured at 30 ºC in YPD media (1% yeast extract, 2% peptone and 2% glucose) or SD (0.67% yeast nitrogen base without amino acids, DIFCO) plus 2% glucose or maltose and supplemented with the appropriate auxotrophic requirements (Sherman *et al*., 1986). Yeast transformants containing the geneticin resistance module (kanMX4) were selected on YPD-agar plates supplemented with 300 µg/L of G418 (geneticin), added after autoclaving and cooling to 60 °C (G418 was used from several suppliers - all work well). LB medium were prepared as previously described (Sambrook J, 1989). When necessary 100 µg/mL of ampicillin was added to standard LB plates or liquid after autoclaving and cooling to 60 °C. A sterile filtered stock solution was used in this case.

Reagents

Oligonucleotides (0.05 µmol scale) were purchased from MWG Biotech, Germany. Restriction and modification enzymes were from Roche Applied Science, Germany. Accuzyme DNA Polymerase was obtained from Bioline, Germany.

Southern blot analysis

Southern blot analysis were performed using a DIG High Prime DNA labelling and detection starter kit II (Roche, Mannheim, Germany) by following the instructions of the supplier. Yeast DNA was obtained according to Homan and Winston (Hoffman & Winston, 1987). Primers used to amplify *Lgt1* probe from genomic DNA are listed in table I. Ten µg of genomic DNA was digested to completion with the restriction enzymes *Sal*I, *Bgl*II, and *EcoR*I and blotted onto Hybond N membrane (Amersham). The membrane was hybridized 3 hours at 42 ºC in the buffer containing the radioactively labelled probe of 518 bp.

Table III - Oligonucleotides used in this study

Primer sequence 5' to 3'	Comments
CCAAGCGCTCTCTATCCAG	*LGT1* probe (southern blot)
CCATCGCTTTGTCTTCTACT	*LGT1* probe (southern blot)
GCGCCCGGGATGTCTACTACAGA	*LGT1* overexpression
GCGCTCGAGTTATTTGGAGAAAA	*LGT1* overexpression
CGCCTGCAGTTGTCCAGACAGCACC	*IGT1* subcloning
CGCGAATTC CCATCTTCCGCCAAGC	*IGT1*subcloning
AATTCAACTCCCGATCTAATATC	*S. cerevisiae HXT1*
TTATTTCCTGCTAAACAAACTCTTGT	*S. cerevisiae HXT1*
TCTGAATTCGCTACTAGCCG	*S. cerevisiae HXT2*
TTATTCCTCGGAAACTCTTTTTTCTT	*S. cerevisiae HXT2*
AATTCAACTCCAGATTTAATATCT	*S. cerevisiae HXT3*
TTATTTCTTGCCGAACATTTTCTTGT	*S. cerevisiae HXT3*
TCTGAAGAAGCTGCCTATCAA	*S. cerevisiae HXT4*
TTAGATCATCAGCGTTGTAGTCAGT	*S. cerevisiae HXT4*
TCACAAGACGCTGCTATTGCA	*S. cerevisiae HXT6/7*
TTATTTGGTGCTGAACATTCTCTTG	*S. cerevisiae HXT6/7*

DNA manipulations and sequencing

Standard DNA manipulations were carried out as previously described (Sambrook J, 1989) (Appendix I). Analysis of sequence data was carried out using DNAMAN sequence analysis software (Lynnon Biosoft). Similarity searches were performed at the National Center for Biotechnology Information, using BLAST software (Altschul *et al.*, 1997). Multiple sequence alignment and phylogenetic analysis were conducted in MEGA4 (Tamura *et al.*, 2007). Search of Igt1p transmembrane regions was carried out by TMHMM 2.0 (Sonnhammer *et al.*, 1998), SOSUI (Hirokawa *et al.*, 1998) and TMPRED (Stoffel, 1993), POLYVIEW (Porollo *et al.*, 2004) and PHOBIUS (Kall *et al.*, 2004). 3D prediction was performed using HHpred Modeller (Eswar *et al.*, 2008) and figures were prepared with program Swiss PDB viewer (Guex & Peitsch 1997).

Construction of *LGT1* disrupted and overexpressing strains

The *LGT1* disruption cassette was obtained by restriction followed by subcloning as described in Pacheco *et al*. 2008. The *Pvu*II- *loxP-KanMX-loxP-Spe*I fragment released by restriction from plasmid pUG6 (Goldstein & McCusker, 1999) was cloned into *Bal*I/*Nhe*I restricted YepHxt6 (a plasmid containing *LGT1* ORF and part of the gene promoter and terminator regions) (Alves-Araújo *et al.*, 2005), creating YLgt1kan. Td*LGT1* disruption cassette which contains the *loxP-KanMX-loxP* module flanked by 320 and 345 bp (5′ and 3′ sides, respectively), homologues to each margin of *LGT1*, was generated

by PCR using the YLgt1kan plasmid as template. Correct disruption of the Td*LGT1* gene was detected by diagnostic PCR using whole yeast cells (Huxley *et al.*, 1990) from isolated colonies and a set of oligonucleotides designed to bind outside or inside of the replaced Td*LGT1* sequence and within the marker module. To obtain a *T. delbrueckii LGT1* overexpressing strain, the *Xma*I-*Lgt1-Xho*I 1750 bp-lengh was PCR-amplified from genomic DNA of *T. delbrueckii* PYCC 5321. Then we cloned the *Xma*I-*Lgt1-Xho*I PCR product into the *Xma*I/*Xho*I restricted and dephosphorilated p426GPD vector (Mumberg *et al.*, 1995), creating pGPDLGT1 vector. The oligonucleotides used are described in table I. *Xma*I/*Xho*I restriction sites were introduced to simplify subcloning.

The 2384 bp *Sac*I-GPD prom-*Lgt1-Xho*I fragment, from pGPDLGT1was cloned on pRS41H centromeric plasmid vector, creating pGPDH. Afterwards the 262 bp *Xho*I-CYC term–*Kpn*I fragment from pGPDLGT1 was cloned on pGPDH creating pGPDLGT1H, which contain the *LGT1* gene under the regulation of the *S. cerevisiae* GPD promoter from p426GPD. DNA fragments resolved in agarose gels were purified by use of a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany).

Subcloning *IGT1* gene

The plasmid YEpIgt1 was generated by cloning a 2760 bp *Pst*I–*Eco*RI fragment from plasmid YEpT-2 (Alves-Araújo *et al.*, 2005), containing the whole *IGT1* gene, 590 bp of the promoter and 497 bp of the terminator into the *Pst*I–*Eco*RI sites of the YEplac181 vector (Gietz & Sugino, 1988). The oligonucleotides used are described in table I. *Pst*I–*Eco*RI restriction sites were introduced to simplify subcloning. DNA fragments resolved in agarose gels were purified by use of a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany).

Yeast transformation

All yeast transformations were performed using the lithium acetate protocol as previously described (Schiestl & Gietz, 1989). Correct yeast transformations were verified, by plasmid DNA isolation using ChargesSwitch plasmid yeast mini kit (Invritrogen, U.S.A) and subsequent transformation in *Escherichia coli* according to SEM method (Inoue *et al.*, 1990). DNA cloning and manipulation were performed according to the standard protocols as described (Sambrook J, 1989). See Appendix I.

Glucose uptake assays

For glucose uptake assays, [U-^{14}C] glucose with a specific activity of 310 mCi/mmol (Amersham) was used. The cells were harvested at exponential phase ($OD_{640\ nm}$ = 0.5–0.6) by centrifugation, washed twice with cold water, and suspended in water to a cellular density of 35–45 mg (biomass dry weight) yeast cells/mL. Glucose uptake assays were performed as described earlier (Alves-Araújo *et al.*, 2005) and radioactivity was measured with a liquid scintillation counter (Packard Instrument Co., Inc.). Glucose uptake was determined with glucose concentrations from 0.1–100 mM.

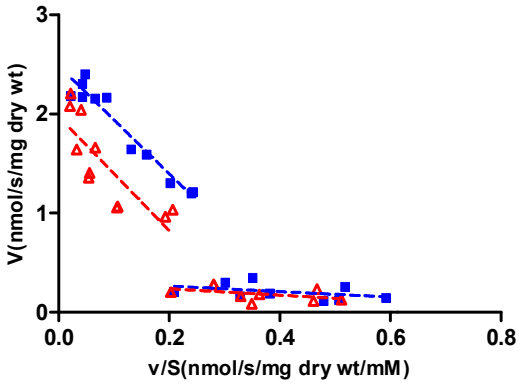
To determine the inhibiting effect of other sugars on glucose transport, competition experiments were carried out by measuring the uptake of glucose at each concentration in the presence of an excess of other unlabelled sugar. The two sugars were added simultaneously to the reaction mixture. Concentration of the inhibitors was 100 mM. Triplicate determinations were performed at each glucose concentration. Kinetic parameters were derived using computer-assisted non-linear regression by using GraphPad Prism software (Microsoft Corp.); however, when the regression was best fitted to two equations, values were obtained from Eadie–Hofstee plots.

Results and Discussion

Glucose transport in *LGT1* disrupted and overexpressing strains

In a previous work we had isolated and characterized *LGT1* gene, the first hexose transporter from *T. delbrueckii.* To further investigate the physiological role of *LGT1*, we created a *T. delbrueckii* Δ*lgt1* mutant by targeted gene disruption (Pacheco *et al.*, 2008). Deletion of this hexose transporter gene resulted in a small decrease of glucose uptake rates, only evident at the higher concentration range. The apparent K_m of the low-affinity component did not change but v_{max} decreased by approximately 20% (fig.1). These results are consistent with the fact that Lgt1p is a low-affinity transporter and point to the existence of other physiologically relevant hexose transporters in this yeast. Also in agreement with these results the Δl*gt1* null strain did not present any growth defect in glucose media. The same has been described for individual *HXT* mutants in *S. cerevisiae,* which did not show any growth phenotype, nor dramatic changes in uptake kinetics (Reifenberger *et al.*, 1995). Additionally, we also constructed an *LGT1* overexpressing strain of *T. delbrueckii* using a plasmid with the dominant *hphNT1* marker gene (pGPDLGT1H). This module plays an important role in selection experiments as it can be used in wild type yeast strains lacking the conventional yeast markers (Guldener *et al.*, 1996). Functionality of Lgt1p from this plasmid was confirmed using a *S. cerevisiae hxt* null mutant which is not capable of growing on glucose, fructose, or mannose (Boles & Hollenberg, 1997). Transformation of the *hxt* null strain with pGPDLGT1H could restore normal growth on glucose. However, *T. delbrueckii* carrying this plasmid did not display any changes in glucose uptake rates, in either affinity component (data not shown).

A



B

Kinetic parameters	Wt low-affinity	Δ*lgt1* low-affinity
K_m (mM)	5.44 ± 0.45	5.74 ± 1.36
V_{max}(nmol/s/ mg dry wt)	2.44 ± 0.06	1.97 ± 0.14

Fig. 1A. Eadie-Hofstee plot of glucose initial uptake rates in cells of *T. delbrueckii* PYCC 5321 (■) and Δ*lgt1* (Δ). **B.** Kinetic parameters of the low affinity component of glucose transport determined for Wt and Δ*lgt1* strains. For glucose uptake assays, [U-^{14}C] glucose with a specific activity of 310 mCi/mmol (Amersham) was used. The cells were grown YPD 2% glucose (w/v), at 30 ºC and harvested at exponential phase (OD_{640} = 0.5–0.6) by centrifugation, washed twice with cold water, and suspended in water to a cellular density of 35–45 mg (biomass dry weight) yeast cells/ml. Radiolabelled glucose uptake was measured as described in Materials and methods.

Screening of hexose transporter homologous genes in *T. delbrueckii*

Since the results suggested the existence of other physiological relevant glucose transporters, besides Lgt1p, Southern blot analysis of *T. delbrueckii* genomic DNA was performed, in order to estimate the number of possible hexose transporter genes present in this yeast. This analysis was carried out under homologous conditions, using as probe a DNA fragment homologous to *T. delbrueckii LGT1* gene and to a conserved region of the 18 *S. cerevisiae* Hxt transporters. The results revealed four (*Bgl*II), eight (*Eco*RI), three (*Sal*I), six (*Eco*RI/*Bgl*II) and eight (*Eco*RI /*Sal*I) bands (fig.2), suggesting the presence of a multigene family of hexose transporters in *T. delbrueckii*, which is consistent with the evidence available for other yeasts.

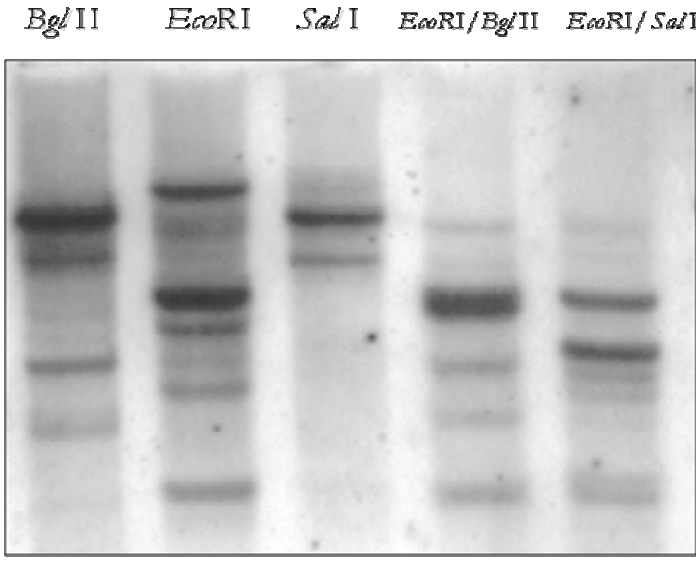


Fig. 2. Southern blot analysis of genomic DNA from *T. delbruecckii* wild type strain. Ten µg of DNA was digested with *Bgl*II, *Eco*RI, and *Sal*I and hybridized with a digoxigenin-labelled DNA probe of 518 bp, under homologous conditions (37ºC).

In the search for other genes involved on glucose transport in *T. delbrueckii*, we screened by PCR, genes homologous to *S. cerevisiae* most

important glucose transporters, *HXT*1 to *HXT4* plus *HXT6* and *HXT7* genes (Boles & Hollenberg, 1997; Ozcan & Johnston, 1999). Using primer pairs (table I) described in *Saccharomyces* Genome Database (SGD), two PCR products were obtained (data not shown). However, the sizes of the PCR fragments obtained were very small ~ 600 bp (with *HXT6/7* primer pair) and ~ 800 bp (with *HXT4* primer pair) when compared to the size of the corresponding *S. cerevisiae*'s *HXT* genes (~1650-1800 bp). Sequencing of the resultant PCR products did not reveal any significant homologies with known transporter genes.

Cloning of *IGT1* gene involved in glucose transport

In a previous work (Alves-Araújo *et al.*, 2005), we transformed the *S. cerevisiae* strain EBY.VW4000 (*hxt* null strain) (Wieczorke *et al.*, 1999), which is deleted for all its hexose transporter genes and is unable to take up and to grow as the sole carbon source on glucose, fructose, mannose, galactose, or sucrose, with a genomic library of *T. delbrueckii* PYCC 5321 (Hernandez-Lopez *et al.*, 2002). The transformants were selected on a leucine free medium with glucose as a sole carbon source and four of these transformants were used to retransform the *S. cerevisiae* mutant strain. All of them were able to confer the ability to grow on glucose and showed mediated glucose transport, with K_m values in the range of 12–25 mM (Alves-Araújo *et al.*, 2005). One of the plasmids, YEpT-2 was further analyzed in this work. DNA sequencing of the insert from this plasmid revealed the presence of a 1673 bp length uninterrupted open reading frame (ORF) showing a high similarity (70–80% of total identity) to formerly reported yeast hexose transporters and 91% of total identity with *LGT1*. Downstream of this *ORF* we also found part of the *LGT1* promotor showing that the two genes are arranged in tandem (fig. 3). This gene disposition had already been described in *S. cerevisiae* for *HXT1*, *4, 5* and *HXT3, 6, 7* arranged in two clusters (Reifenberger *et al.*, 1995). We named the new gene *IGT1*, for intermediate glucose transporter.

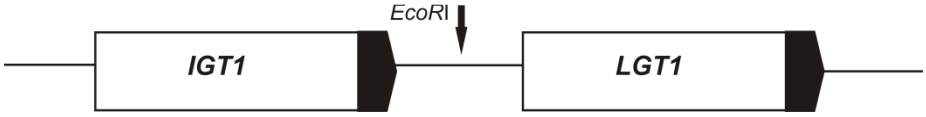


Fig. 3. Genetic organization map of *T. delbrueckii* cluster containing *IGT1* and *LGT1* genes. Direction of open reading frames is indicated by arrows. *EcoRI* restriction site is indicated.

Characterization of the *IGT1* gene

Analysis of the *IGT1* upstream sequence (1000bp upstream from the ATG) showed the presence of several Rgt1p and Mig1p-binding sequences. The transcriptional factors Mig1p and Rgt1p have been reported as required for the transcriptional regulation of hexose transporters in *S. cerevisiae.* Mig1p mediates the repression at high concentrations of glucose of *HXT2* and *HXT4* expression, while Rgt1p acts as a bifunctional regulator of *HXT1–HXT4* (Kim *et al.*, 2003; Ozcan & Johnston, 1996). We mapped 7 sequences in both orientations matching the Rgt1p consensus binding site 5-

CGGANNA-3 (Kim *et al.*, 2003), all distributed in clusters, −209 to −240 (two sites), −439 to −503 (three sites) and −663 to −711 (two sites). The occurrence of multiple Rgt1p binding sites distributed in clusters has previously been reported in the promoters of *HXT* genes of *S. cerevisiae* (Kim *et al.*, 2003) as well as of *T. delbrueckii LGT1* gene (Alves-Araújo *et al.*, 2005). In this gene Rgt1p appears to act exclusively as a repressor of expression, and not as a bifunctional regulator (Alves-Araújo *et al.*, 2005). We also found in the promoter of *IGT1* seven sequences, containing a perfect Mig1p-binding GC box, (G/C)(C/T)GGGG (Nehlin & Ronne, 1990), however only five contain a 5' AT-rich region adjacent to the GC box. Furthermore, we mapped seven Gcr1p binding sequences (6 sequences in the forward strand and 1 sequence in the reverse strand), a protein that has been described as required for the regulated high level transcription of glycolytic genes, and was found to regulate transcription of the *S. cerevisiae HXT4* gene (Turkel & Bisson, 1999). Sequence analysis of the region upstream of *IGT1* also showed the presence of a potential TATA box at position −132, from the ATG codon. These elements are often found in yeast promoters, playing a critical role in transcription (Struhl, 1982).

Sequence characterization of Igt1p

The putative ORF from *IGT1* codes for a protein with 553 amino acids (fig. 4). As expected, significant similarities were found between the amino acid sequence predicted from *IGT1* and a large family of other yeast hexose transporters (fig.5). This similarity is even greater when compared to Lgt1p the only hexose transporter described in *T.delbrueckii* (fig. 4 and 5). The Hxtp family of *S. cerevisiae* (61–78% identity) is the one with higher similarities among other yeast transporters (fig. 5).

```
Igt1    1    MSTQENTPVGHLTPRASGSHSVLSTPSNKAERDDAKDLNSMAAPEPAIDIPKRPASSYIG  60
Lgt1    1    ...T.H...D..S.AV.A.G.G............I..FDAA.DR.A.V............  60

Igt1    61   VSILCLMVAFGGFVFGWDTGTISGFVNLSDFLKRFGQRNSEGEYYLSKVRMGLIVSIFNI  120
Lgt1    61   ................................Q.....SKD.V.................  120

Igt1    121  GCAIGGLVLSKIGDVYGRRYWFGCCHCHLCRWYLDSNLFFRQMVPILIGRIISGLGVGGI  180
Lgt1    121  ......II......I....IGLVAVTVIYVVGI.IQICS.NKWYQYF.............  180

Igt1    181  AVLSPMLISEVSPKQIRGTLVACYQLMITLGIFLGYCTNYGTKTYDDSTQWRVGLGLCFA  240
Lgt1    181  .............................................N..............  240

Igt1    241  WAIFMIAAMFFVPESPRYLVEVGNFEEAKRSLSRSNKVSVDDPALLAELDAISAGVEAER  300
Lgt1    241  ......GG.L..........H..I...........L......V.............K  300

Igt1    301  LAGNASIGELFSTKTKVFQRLIMGVMLQSLQQLTGDNYFFYYGTTIFKSVGLKDSFQTSI  360
Lgt1    301  ...S..W.....................................V..............  360

Igt1    361  IIGVVNFFSTFVGIYCIERFGRRTCLLWGSATMVCCFVVFASVGVTKLWPEGPNHQDISS  420
Lgt1    361  ..............F.............A.S.....................S......  420

Igt1    421  KGAGNCMIVFTMFYIFCFATTWAGGCYVIVSESFPLRVKSKGMAIATGANWLWGFLISFF  480
Lgt1    421  ............F......S.........................A.........A..  480

Igt1    481  TPFITGAINFYYGYVFMGCLVFSFFYVFFFVPETKGLTLEEVNTMWLEGVLPWKSASWVP  540
Lgt1    481  ......................GY..................E.............A...  540

Igt1    541  PDRRGADYNAEEMAHDD  557
Lgt1    541  .................  557
```

Fig. 4. Amino acid sequence alignment between the *T. delbruecckii* proteins Lgt1p and Igt1p. Residues that are similar in both proteins are marked with a point and the mismatched residues are specified. The numbers mark the amino-acid positions. Identities = 481/557 (86%). Amino acids abbreviations are identified on the Abbreviation section.

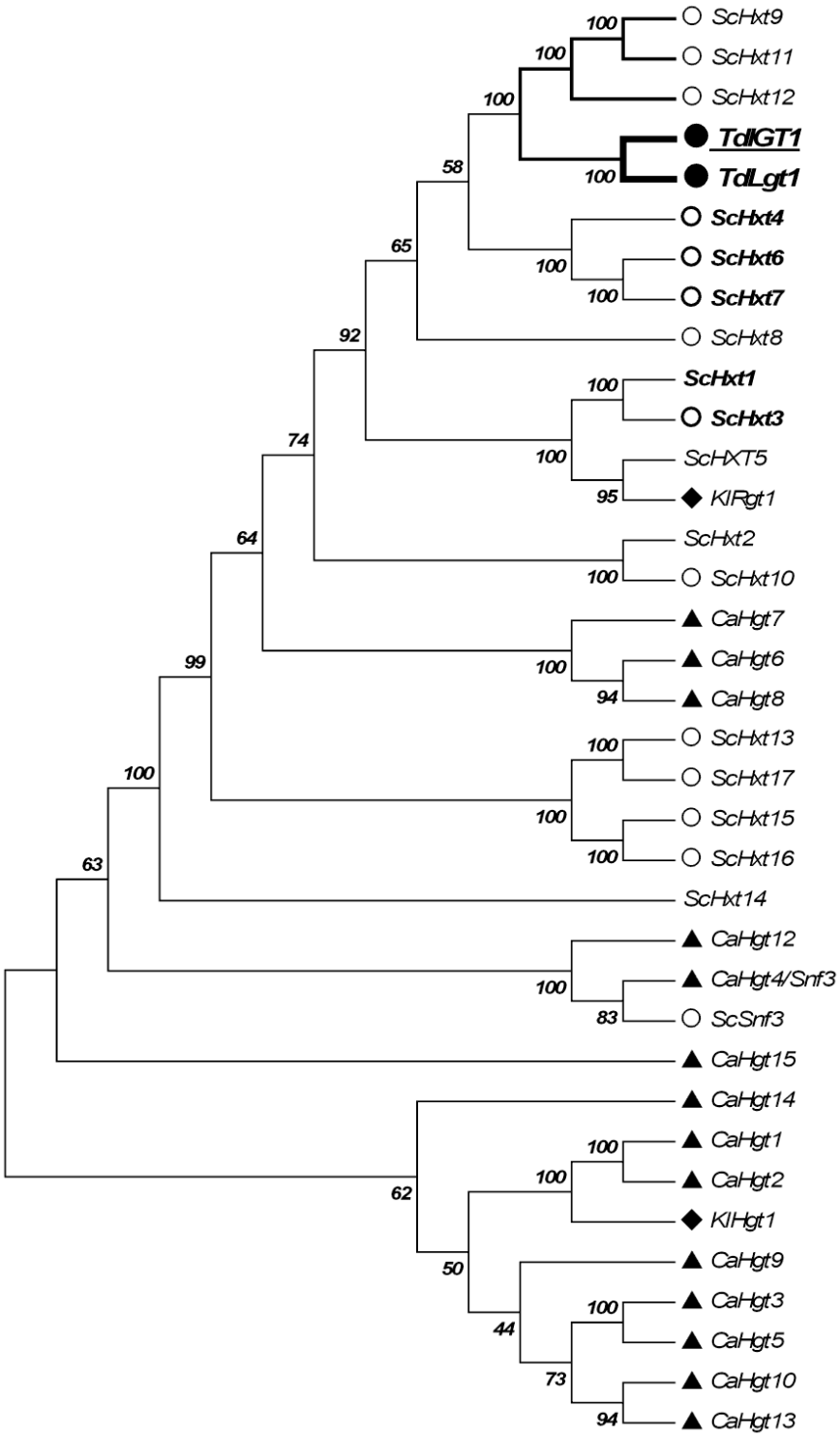


Fig. 5. Relationships between Igt1p (underlined) and other yeast hexose transporters. *T. delbrueckii* (●) Igt1p and Lgt1p appear in larger and bold letters. The *S. cerevisiae* (○) Hxt1, Hxt2, Hxt3, Hxt4, Hxt6 and Hxt7, considered the major hexose transporters in this yeast, are indicated in small bold letters. *Candida albicans* (▲) and *Kluyveromyces lactis* (◆) are also specified. The evolutionary tree was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

For the later, the highest similarity was observed for Hxt9p/Hxt11p (75%), two genes not regulated by glucose, which play a role in drug resistance (Nourani *et al*., 1997), for Hxt12p, which is almost identical to Hxt11p, and for Hxt6p/Hxt7p (73%) and Hxt4p (71%) encoding high- and intermediate affinity transporters, respectively.

There are many transmembrane (TM) helix prediction tools. Although most of them can predict topologies reasonably well (Hirokawa *et al*., 1998; Porollo *et al*., 2004; Sonnhammer *et al*., 1998; Stoffel, 1993) the prediction accuracy for the N-terminus region is only 50–70% (Ikeda *et al*., 2002; Melen *et al*., 2003). This is critical to accurately predict TM helix numbers and positions, because a mis-predicted TM helix can reverse the TM topology of a downstream region of the protein. The putative Igt1p membrane topology deduced using several programs, was predicted to have 10 membrane-spanning domains, by TMHMM 2.0 (Sonnhammer *et al*., 1998), SOSUI (Hirokawa *et al*., 1998) and TMPRED (Stoffel, 1993), 11 by POLYVIEW (Porollo *et al*., 2004) and 12 by PHOBIUS (Kall *et al*., 2004)(Table II).

Table II - Igt1p transmembrane helix boundaries predicted by several prediction programs.

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
TMHMM 2.0	55-77	110-130		166-188	198-220	233-252		355-377	384-406	428-450	462-484	488-510
SOSUI	54-76	110-132		165-187	197-219	233-255		356-378	386-408	427-449	469-491	494-516
PHOBIUS (POLYPHOBIUS)	57-77	110-129	140-154	163-188	200-221	233-252	321-340	358-377	385-405	427-250	462-484	490-511
POLYVIEW	57-88	108-133	139-158	163-190	196-223	230-251	317-349	355-379	385-407	422-447	458-486	491-509
TMPRED	58-77	111-130		171-188	200-217	233-251		358-377	385-405	427-450	462-488	490-509

We believe that the prediction given by PHOBIUS is the more truthful not only because PHOBIUS was previously described as one of the most accurate TM helix predictor (Lee *et al*., 2006) but also because the presence of 12 putative transmembrane domains (TMD) in the hexose transporters is highly conserved, as these proteins differ the most in the length and sequence of the cytosolic amino- and carboxyl- terminal regions (Kruckeberg, 1996). Supporting this interpretation, the 3D modelling of *IGT1* using *E. coli* lactate permease transporter template, also predicted the presence of 12 TMD (fig. 6).

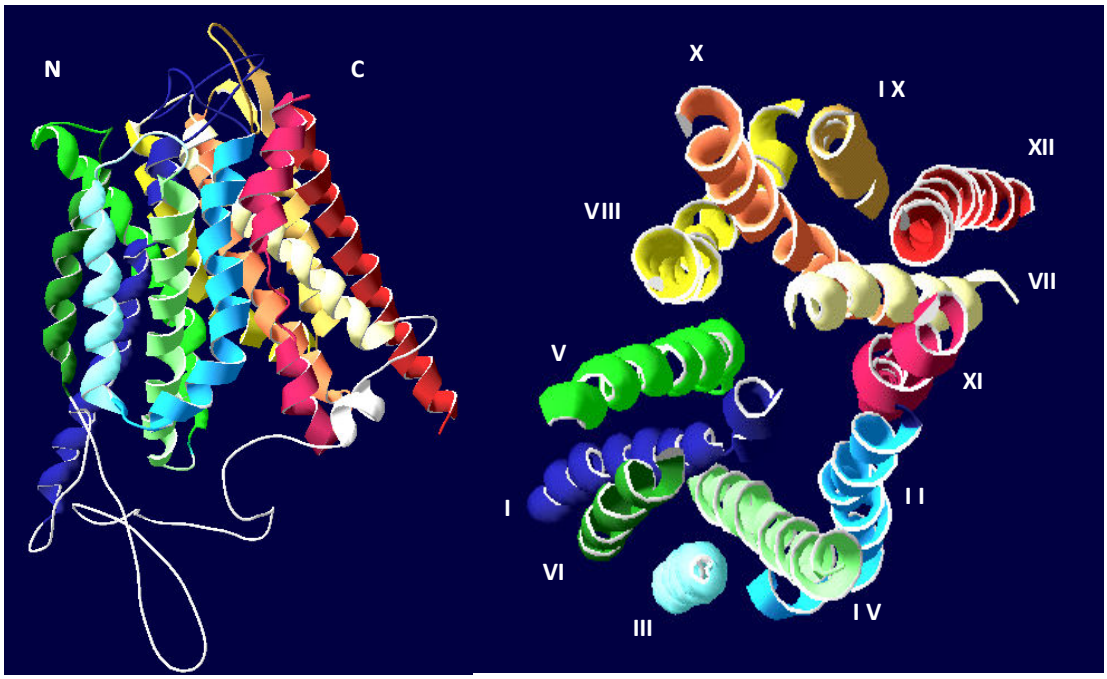


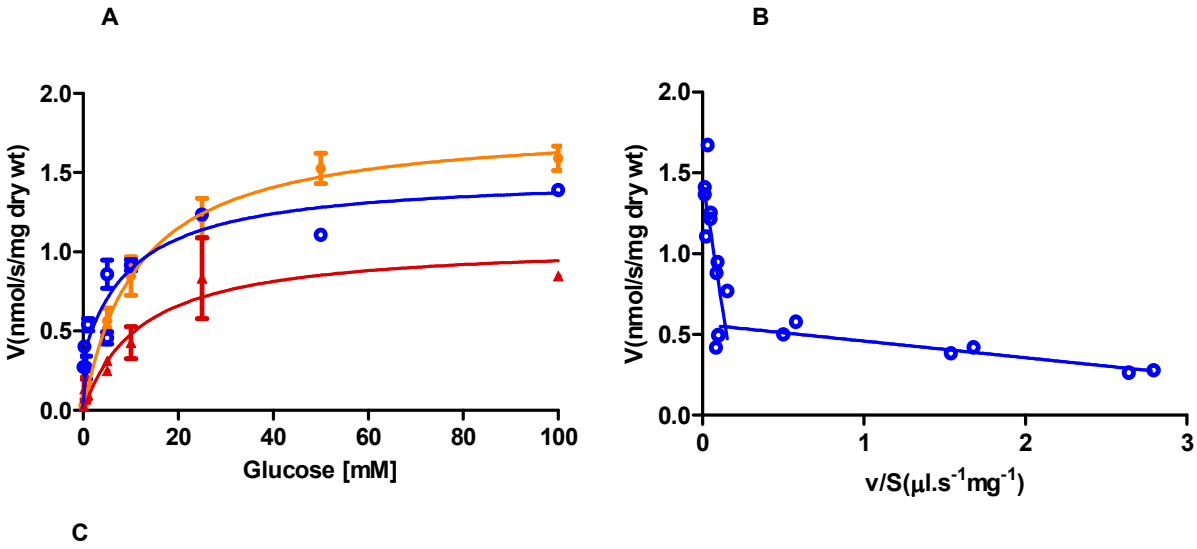
Fig. 6. Ribbon representation of Igt1p structure modelled to the LacY permease (HHpred Modeller), (left) View parallel to the membrane; (right) View from the cytosol side (the loop regions are not represented). Transmembrane domains are indicated from I to XII and from N to C terminus. Figures were prepared with program Swiss PDB viewer (Guex & Peitsch 1997).

When compared with transporter proteins that are predicted to have 12 transmembrane domains, the 3th and 7th domains are missing in the 10 TMD predictions of Igt1p. Indeed, these regions contain a few more hydrophilic residues not reaching the threshold hydrophobicity required to specify it as a transmembrane domain in some programs. Nonetheless, transmembrane domains 3 and 7 were predicted to be involved in substrate binding or substrate translocation across the membrane (Kruckeberg, 1996; Ozcan & Johnston, 1999), and this could mean a lower contact with the lipid bilayer.

Kinetic characterization of glucose transport in the *IGT1*- transformed *S. cerevisiae hxt* null strain

Kinetic characterization of glucose transport was performed with the strain *S. cerevisiae* EBY.VW4000 transformed with the plasmid YEpIgt1, generated by cloning a 2760 bp *Pst*I–*Eco*RI fragment from plasmid YEpT-2 (Alves-Araújo *et al*., 2005) which contains the whole *IGT1* gene, 590 bp of the promoter and 497 bp of the terminator. Glucose uptake was measured in the transformed cells and, as a control, in cells harbouring the empty vector YEplac181. Control cells were grown in SD-maltose (OD_{640} = 0.2–0.3) and transferred to SD-glucose for 4 h. Under these conditions the control strain did not exhibit any measurable glucose uptake. Introduction of *IGT1* gene into the *hxt* null strain was sufficient to allow it to grow on glucose, fructose and mannose (2%, w/v). Growth on glucose was also observed at 0.1% and 4% glucose, suggesting that Igt1p is expressed and enables transport at both high-and low glucose concentrations. Cells of *S. cerevisiae hxt* null strain transformed with *IGT1,*

grown in SD-glucose 2% (OD_{640} = 0.5–0.6), exhibited carrier-mediated glucose transport best fitted assuming a Michaelis–Menten kinetics (fig. 7A) with an apparent K_m value of 11.5±1.5 mM, in the range of the intermediate-affinity component, and a V_{max} of 1.81±0.08 nmol/s/mg dry wt (fig. 7C). Surprisingly, when *IGT1* expressing cells are grown in 0.1% glucose, glucose transport best fitted a biphasic Michaelis–Menten kinetics, with an intermediate (K_m = 6.5± 2.0 mM) and a high-affinity (K_m =0.10 ±0.01 mM) component (fig. 7B).



C

Kinetic parameters	Glu 0.1%		Glu 2%	Glu 4%
	High-affinity component	Intermediate-affinity component	Intermediate-affinity component	Intermediate-affinity component
K_m (mM)	0.10 ± 0.01	6.5 ± 2.0	11.5 ± 1.5	12.2 ± 2.6
V_{max} (nmol/s/mg dry wt)	0.56 ± 0.02	1.50 ± 0.15	1.81 ± 0.08	0.90 ± 0.09

Fig. 7A. Michaelis-Menten plot of glucose initial uptake rates in cells of *S. cerevisiae hxt* null strain transformed with the *IGT1* gene. Cells were grown in SD 0.1 % (○) 2% (●) and 4% (▲) glucose without leucine, and harvested during exponential growth (OD_{640} = 0.5–0.6). **B.** Eadie–Hofstee plots of glucose initial uptake rates in cells of *S. cerevisiae hxt* null strain transformed with the *IGT1* gene. Cells were grown in SD 0.1% glucose (○) without leucine,and harvested during exponential growth (OD_{640} = 0.5–0.6). **C** Kinetic parameters of glucose initial uptake in the cells described above. Where the data fitted more accurately to two transport components, the kinetic parameters of both components are given. Radiolabelled glucose uptake was measured as described in Materials and methods.

However SD 4% glucose-grown cells exhibited again a kinetics of glucose transport best fitting a low affinity component with an apparent K_m =12.2 ±2.6 mM, in the range of the intermediate-affinity component, and a V_{max} of 1.06 ±0.16 nmol/s/mg dry wt (fig. 7C). Although not frequent among hexose

transporters this behaviour is similar to what has been described for *S. cerevisiae* Hxt2p (Reifenberger *et al.*, 1997). In agreement with the growth assays, glucose transport was competitively inhibited by fructose (fig. 8), indicating that this sugar shared the Igt1 transporter. In presence of 100 mM fructose the apparent K_m increased to 44.2± 9.6 mM, v_{max} remaining constant. The presence of 100 mM mannose only slightly inhibited the glucose transport, K_m and V_{max} values remaining almost unaltered. This result, together with the growth assays suggests that this protein transports mannose although with a very low affinity.

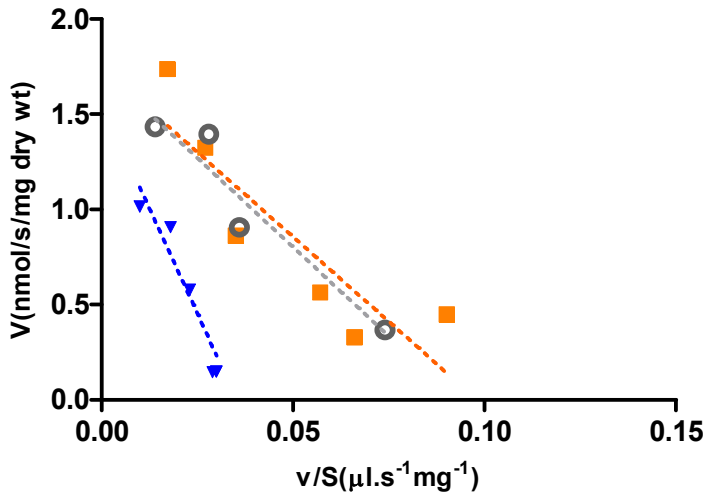


Fig. 8. Eadie–Hofstee plots of glucose initial uptake rates in the presence of other sugars in cells of *S. cerevisiae hxt* null strain transformed with the *IGT1* gene. SD-maltose (without leucine)- grown cells were washed with chilled water, and transferred (OD640 = 0.5–0.6) to SD medium containing 2% glucose without leucine. Zero *trans*-influx of [U-^{14}C]glucose was measured in the absence of glucose (■), and presence of 100 mM mannose (○) or 100 mM fructose (▼). Radiolabelled glucose uptake was measured as described in materials and methods.

Final considerations

Many investigations have been performed on the molecular genetics of hexose transport in several yeast species, such as *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Pichia stipitis* (Boles, 2002). Nevertheless, on a molecular level, very little information is available on hexose transport in the as well non-conventional yeast *Torulaspora delbrueckii*. In this study we searched for hexose transporter genes in *T. delbrueckii* PYCC 5321 by three approaches. First we used PCR screening with *S. cerevisiae HXT1* to *HXT4* plus *HXT6* and *HXT7* (Boles & Hollenberg, 1997; Ozcan & Johnston, 1999) primer pairs, however we could not find any homologous transporter genes. Then from a genomic library of *T. delbrueckii*, we recovered several plasmids that could complement the glucose growth defect of a hexose transport-null strain of *S. cerevisiae,* supporting the occurrence of several hexose transporters. This method resulted in the formerly isolation of *LGT1*, the first gene in *T.delbrueckii* involved in glucose transport (Alves-Araújo *et al.*,

2005). One other of those plasmids was further investigated and here we describe the presence in *T. delbrueckii* of a second putative membrane protein with a deduced secondary structure sharing several features with members of the Major Facilitators Superfamily (MFS) (Marger & Saier, 1993). This gene was named *IGT1* (intermediate glucose transporter). Best protein sequence similarity has been found with the functionally characterized monosaccharide transporter of *T. delbrueckii* Lgt1p (Alves-Araújo *et al.*, 2005), but a significant similarity was also displayed throughout its entire sequence with other putative ascomycetes hexose transporters, especially with *Kluyveromyces lactis* Kht2p and Rag1p (64–72%), and for the Hxtp family of *S. cerevisiae* (61–75%). Despite the high nucleotide and protein homology with *T. delbrueckii* Lgt1p and with *K. lactis* Kht2p, Igt1p presents different regulation and transport properties. Kinetic studies in *S. cerevisiae* suggest that Igt1p is closely related to the *S. cerevisiae* Hxt2 transporter. Uptake kinetics obtained for *IGT1* expressed in the *hxt-null S. cerevisiae* strain EBYVW.4000 demonstrate that individual Igt1 protein can function independently as hexose transporter, enconding an intermediate affinity glucose transporter. Glucose uptake kinetics was, however, dependent on the growth conditions of the cells. After growth on 2% glucose the apparent Km value for glucose was about 11 mM. In contrast, glucose uptake of cells grown on low glucose concentrations (0.1 % glucose) proved to be non-linear and showed biphasic uptake kinetics with a intermediate and a high-affinity component. Consistent with the finding of a high-affinity component, Igt1p could restore growth of the *hxt* null strain even on 0.1% glucose. On the other hand, transport capacity was decreased in cells grown in 4% glucose suggesting that it is repressed for high glucose concentrations. A similar represion has also been reported for *S. cerevisiae* Hxt2p (Reifenberger *et al.*, 1997). Properties of the Igt1p seem to be modulated in response to different external glucose concentrations, and it may by responsible for transporting glucose when it is scarce. Nevertheless it is likely that *T. delbrueckii* has other high affinity transporters further than Igt1p. The existence of other high affinity transporters is supported by the kinetics of glucose transport in *T. delbrueckii* cells grown in 2% glucose, which displays a mediated glucose transport activity best fitted assuming a biphasic Michaelis–Menten kinetics with a low- and a high affinity component, not displayed by Igt1p when cells are grown at this glucose concentration. Yet since data on the kinetics of glucose transport mediated by *IGT1* was obtained in a *S. cerevisiae* strain, the results may not necessarily reflect the *in vivo* function of this transporter. Also consistent with the existence of several hexose transporters, the estimate of the number of homologous genes by Southern blots analysis of this yeast genome, with a probe homologous to *LGT1* gene, revealed the existence of several bands. Since *LGT1* and *IGT1* genes are located in tandem (fig. 3) and are not cut by *Bgl*II or *Sal*I, and *Eco*RI cuts only once between the two genes, the paterns found suggest that there are other hexose transporter homologous genes, other than *LGT1* and *IGT1*. Consistently, disruption of *LGT1* resulted in a small decrease of glucose uptake rates, most clear at the higher concentration range, and did not present any growth defect in glucose media.

All together these evidences indicate the existence of several hexose transporters with different glucose affinities and regulation. Yeast adapts to the concentration of hexoses in its environment by expressing a transport system that has a substrate affinity appropriate to those conditions. As a result the presence of multiple hexose transporters with different affinities for glucose

in *T. delbrueckii* PYCC 5321 is not surprising, given the fact that its natural environment is bread dough and is able to grow well on a broad range of glucose concentrations (Alves-Araújo *et al.*, 2007).

Acknowledgements

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Chapter 6

Small heat shock protein Hsp12p contributes to yeast tolerance to freezing stress

This chapter comprises parts from the following publication:

Pacheco A., Pereira C., Almeida MJ. & Sousa MJ. (2008). Small heat shock protein Hsp12p contributes to yeast tolerance to freezing stress. Submitted manuscript.

Abstract

HSP12 gene encodes one of the two major small heat shock proteins of *Saccharomyces cerevisiae* and is induced under different conditions, such as low and high temperatures, osmotic or oxidative stress and high sugar or ethanol concentrations. However, few studies could demonstrate any correlation between *HSP12* deletion or overexpression and a phenotype of sensitivity/resistance, making it difficult to attribute a role for Hsp12p under several of these stress conditions. Herein, we investigated the possible role of Hsp12p in yeast freezing tolerance. Contrary to what would be expected, the *hsp12* null mutant when subjected to prolonged storage at -20 °C showed an increased resistance to freezing when compared with the isogenic wild type strain. Because the mutant strain displayed a higher intracellular trehalose concentration than the wild type, which could mask the effect of manipulating *HSP12*, we overexpressed *HSP12* gene in a trehalose-6-phosphate synthase *(TPS1)* null mutant. Δ*tps1* strain overexpressing *HSP12* showed an increase in resistance to freezing storage, indicating that Hsp12p plays a role in freezing tolerance in a way that seems to be interchangeable with trehalose. In addition, we show that overexpression of *HSP12* in this Δ*tps1* strain also increased resistance to heat shock and that absence of *HSP12* compromises the ability of yeast cells to accumulate high levels of trehalose in response to a mild heat stress.

Introduction

Because freezing is one of the major abiotic stresses, the adaptation mechanisms that preserve cells at subzero temperatures are extremely important in the development of technology for the cryopreservation of life. Preservation of cell activity is also a very important issue in frozen-dough technology. This process is well established in the modern baking industry, as it can more easily supply oven-fresh bakery products to consumers while improving labour conditions. Nevertheless, storage of frozen bread dough may lead to the loss of baker's yeast cell viability as well as of its baking capacity, and consequently to economic losses (Alves-Araújo *et al.*, 2004a; Randez-Gil *et al.*, 1999). Despite its importance, limited information is available about the mechanisms and determinants of freezing resistance and cold responses in yeast (Kandror & Goldberg, 1997; Kandror *et al.*, 2004; Odani *et al.*, 2003; Zarka *et al.*, 2003). When yeast cells are cultured at 4 ºC for a long term, several heat shock proteins (HSPs) are induced (Homma *et al.*, 2003), suggesting that the induction of these genes might be necessary for adjustment to cold resistance. Considerable evidence indicates that the intracellular level of trehalose may determine the survival response of yeasts under extreme environmental conditions (Diniz-Mendes *et al.*, 1999; Hottiger *et al.*, 1987; Singer & Lindquist, 1998; Van Dijck *et al.*, 1995; Wiemken, 1990). In general, there is wide consensus that trehalose can serve as a stress protectant when yeast cells are confronted with high temperatures (Attfield, 1987; Hottiger *et al.*, 1987). On the other hand, no direct correlation was observed between trehalose accumulation and freezing resistance, above a certain intracellular concentration (Alves-Araújo *et al.*, 2004a).

Small heat shock protein 12 (Hsp12) has been described as a LEA like protein in *S. cerevisiae* (Mtwisha *et al.*, 1998) and it has been reported to be localized both at the plasma membrane, protecting membranes from desiccation (Sales *et al.*, 2000), and in the cytoplasm and cell wall, enhancing barotolerance of the yeast (Motshwene *et al.*, 2004). *HSP12* is induced massively in yeast cells exposed to heat shock, osmostress, oxidative stress, high concentrations of alcohol, as well as in early-stationary-phase cells (Praekelt & Meacock, 1990; Stone *et al.*, 1990). It is also induced at 0 ºC as part of the near-freezing response (Kandror *et al.*, 2004) and by 4 ºC exposure (Murata *et al.*, 2006). However, to date few studies could demonstrate any correlation between *HSP12* disruption or overexpression and a phenotype of sensitivity/resistance, making it difficult to attribute a role for Hsp12p under most of these stress conditions. In this study we investigated the possible role of Hsp12p in freezing tolerance, using the yeast *S. cerevisiae* as a model. We report here that Hsp12p plays a role in cryoresistance, even though the *hsp12* null mutant revealed to be more resistant to freezing than the wild type strain. We also show that stationary-phase cells of the Δ*hsp12* mutant have a higher intracellular trehalose concentration than wild type cells, even though heat-induced trehalose accumulation is impaired in this mutant. Overexpression of *HSP12* in a Δ*tps1* strain allowed to demonstrate a clear increase in resistance to freezing storage and also to heat stress.

Materials and Methods

Strains

Saccharomyces cerevisiae BY4742 wild-type strain and isogenic mutants used in this study are listed in table I. The plasmid vector pRS 41H (Taxis & Knop, 2006) was kindly provided by Christof Taxis and Michael Knop from EMBL Cell Biology and Biophysics Unit, Meyerhofstr 1, D-69117 Heidelberg, Germany.

Media and Growth Conditions

YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose) and LB medium were prepared as previously described (Sambrook J, 1989). For the selection of *hphNT1,* 300 mg/L hygromycin B was added to standard YPD plates or liquid YPD medium after autoclaving and cooling to 60 °C (Duchefa Biochemie, Netherlands). The hygromycin B stock solution was used as provided by the manufacturer. When necessary, 100 µg/mL of ampicillin was added to standard LB plates or liquid medium, after autoclaving and cooling to 60 °C. A sterile filtered stock solution was used in this case. Yeast cells were routinely grown on YPD medium at 30 °C and 200 rpm.

Table I. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4742 (wt)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
Δ*hsp12*	BY4742; YFL014w::kanMX4	EUROSCARF
Δ*tps1*	BY4742; YBR126c::kanMX4	EUROSCARF
Δ*dur1,2*	BY4742;YBR208c::kanMX4	EUROSCARF
Δ*tps1*- p_{empty}	Δ*tps1* pRS41H	this study
Δ*tps1*- p_{HSP12}	Δ*tps1* pRSHSP12	this study

Reagents

Oligonucleotides (0.05 µmol scale) were purchased from MWG Biotech, Germany. Restriction and modification enzymes were from Roche Applied Science, Germany. Accuzyme DNA Polymerase was obtained from Bioline, Germany.

Plasmids construction

The *HSP12* gene sequence was PCR-amplified with the primers Hsp12Fw (5´GATCGGATCCAAATGTCTGACGCAGGTAGAAAAGG3´) and Hsp12tRw (5´GATCGTCGACTTAC

TTCTTGGTTGGGTCTTCTT 3´) from genomic DNA of *S. cerevisiae* BY4742.

The PCR protocol involved an initial denaturation at 94 ºC (5 min), followed by 30 cycles of 30 s at 94 ºC, 30 s at 55 ºC and 1 min at 72 ºC. The last cycle was followed by a final extension step of 10 min at 72 ºC. About 100 ng of DNA template was used in 50 µL reaction mixture. The 300 bp-lengh PCR product was cloned into pGEM T-easy cloning vector (Promega, Madison, WI). The *EcoR*I restricted fragment was then cloned into the *EcoR*I restricted and dephosphorilated p426GPD vector (Mumberg *et al.*, 1995). The 1240 bp *Kpn*I-GPD promoter-HSP12-CYC terminator–*Sac*I fragment was subcloned on pRS41H centromeric plasmid vector creating pRSHSP12, which contains the HSP12 gene under the regulation of the *S. cerevisiae* GPD promoter from p426GPD. DNA fragments resolved in agarose gels were purified by use of a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany).

Escherichia coli transformation

All the vectors constructed were first established in *E. coli* XL1-Blue strain, according to SEM method (Inoue *et al.*, 1990) (Appendix I). The correct plasmid constructs were verified by restriction map analysis followed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer, using the method of Sanger (Sanger & Coulson, 1975).

Yeast transformation

All yeast transformations were performed using the lithium acetate protocol as previously described (Schiestl & Gietz, 1989). Correct yeast transformations were verified by plasmid DNA isolation using ChargesSwitch plasmid yeast mini kit (Invritrogen, U.S.A) and subsequent transformation in *E. coli* according to SEM method. DNA cloning and manipulation were performed according to the standard protocols as described (Sambrook J, 1989). (Appendix I)

Yeast freezing

Freezing assays were performed as previously described (Alves-Araújo *et al.*, 2004a) with minor changes. Twenty five mL of stationary phase cells (24 h of culture) were washed twice with deionised water, and suspended in a quarter of the initial volume in sterile water. Aliquots (500 µL) of cells were transferred into 1.5 mL tubes, centrifuged and the pellet suspended in 100 µL of LF medium (liquid fermentation medium) (Hino *et al.*, 1990). The samples were frozen at -20 ºC for different time periods.

Extraction and assay of trehalose

Trehalose extraction was performed for the yeast suspensions prepared as described above, immediately before freezing, and sampled for dry weight. Cells were harvested by centrifugation and

washed twice with cold deionised water. Trehalose was extracted from cold cell pellets with 5% (w/v) trichloroacetic acid (Merck, Darmstadt, Germany) for 45 min with occasional shaking. Cells were then centrifuged at 735 g, for 10 min. Extraction was repeated once more, and supernatants from the two extractions were combined and used for the determination of trehalose by high-performance liquid chromatography. The apparatus used was a Gilson chromatograph (132-RI Detector), equipped with a carbohydrate H^+ column (SS-100, H^+, Hypersil) that was maintained at 60 ºC. A solution of H_2SO_4 (0.0025 M) was used as the mobile phase at a flow rate of 0.45 mL min^{-1}.

Measurement of cell viability

Cell viability was analyzed by flow cytometry using the membrane exclusion dye propidium iodide (PI), as previously described (Alves-Araújo *et al.*, 2004a). In these assays, cells with conserved membrane integrity are not permeated by propidium iodide (PI^- cells), while those that lost their membrane integrity do incorporate the fluorochrome (PI^+ cells) (Prudencio *et al.*, 1998). Previous results showed that in cells subjected to freezing stress, the loss of cell proliferative capacity (expressed as CFU counts) perfectly correlates with loss of membrane integrity (Alves-Araújo *et al.*, 2004a).

Expression Analysis by Quantitative Real-Time PCR

Relative expression (R.E.) was calculated as 2– ((gene of interest ΔCt) – (calibrator $\Delta^{Ct)}$. Δ Ct was calculated as (sample Ct) – (house keeping gene 18S Ct). The Ct values of both the calibrator and the samples of interest are normalized to the endogenous housekeeping gene 18S. Target gene expression is considered increased if relative expression is >1, once values of R.E. in each experiment are normalized with respect to the calibrator sample, with a R.E. value set to 1. Both actin (ACT1) and 18S transcript levels were used to control for RNA amount. We have found 18S mRNA to be a better control, since the levels of actin mRNA were less constant in cells as already described by others (Seymour & Piper, 1999; Wenzel *et al.*, 1995).

Hsp12p detection

Protein extraction was performed as previously described (Mtwisha *et al.*, 1998) with some modifications. Yeast cells of the BY4742 wild-type (wt) strains and BY4742 Δ*hsp12* strain were grown in YPD at 30 °C, and cells of BY4742 and BY4742 Δ*hsp12* strains transformed with the plasmids pRS41H (control) and pRSHSP12 (overexpressing *HSP12*) were grown in YPD supplemented with 300 mg/L hygromycin, all harvested in stationary phase (*D.O.*= 3-4). Proteins were separated by SDS-PAGE using a Tris-SDS-glycine buffer system (Laemmli, 1970). SDS-PAGE gel images were acquired with Molecular Imager ChemiDoc XRS system and Quantity One software from Biorad. For detailed procedures see Appendix II.

Reproducibility of the results

All the experiments were repeated at least three times, and the data reported are mean values and SD. When statistical analyses were performed, the significance was tested by Anova and T test (GraphPad Prism 5).

Results

∆*hsp12* strain reveals to be more resistant to freezing at - 20 ºC than the wild type strain

Aiming at the investigation of the possible role of Hsp12p in yeast freezing tolerance, we subjected stationary-phase cells of *Sacharomyces cerevisiae* BY4742 (wild type) and of a *hsp12*-deleted isogenic strain to prolonged storage at -20 °C. Since we have previously shown that in cells subjected to freezing stress, loss of cell proliferative capacity perfectly correlated with the loss of membrane integrity (Alves-Araújo *et al.*, 2004a), viability of the cells along freezing was assessed by flow cytometry using the fluorochrome propidium iodide (PI). In both strains, we analyzed cell viability for a storage period of 8 days. Contrary to what would be expected taking into account that *HSP12* is highly induced by cold stress (Kandror *et al.*, 2004; Murata *et al.*, 2006), viability loss during freezing storage at –20 °C was lower for the *hsp12* null mutant than for the wild type strain (fig. 1A). A decrease of 80% in cell viability was observed for the wild type strain after 8 days of storage, whereas ∆*hsp12* strain presented a loss in cell viability of only about 40% after the same period, indicating that the absence of Hsp12p resulted in a higher resistance to freezing. To further support that the observed differences were due to absence/presence of Hsp12p in the cells, the same experiments where performed with cells harvested in exponential growth phase. At this culture stage, *HSP12* expression is repressed. As a consequence, Hsp12p is absent or vestigial in both ∆*hsp12* and wild type strains (Praekelt & Meacock, 1990). As expected, the two strains showed a similar behaviour, no significant differences being observed in freezing resistance at -20 ºC (fig. 1B).

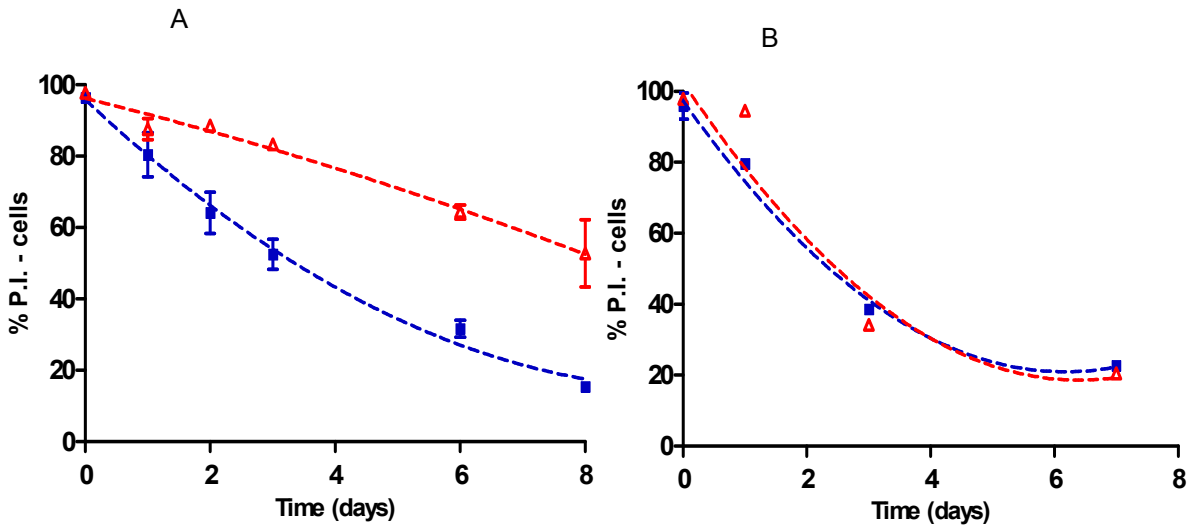

Fig. 1. Cell viability analysis of the wild type (■) and Δhsp12 (Δ) strains in stationary growth phase (**A**) and exponential growth phase (**B**), after freezing at -20 °C. Cells of the BY4742 (wt) and corresponding mutant strain, Δhsp12, were grown in YPD at 30 °C until exponential growth phase (OD_{640nm} ≈ 0.6) (fig.1B) and stationary growth phase (OD_{640nm} ≈ 3-4) (fig.1A), and then subjected to prolonged storage at -20°C. Viability of the cells along freezing was determined by propidium iodide exclusion as measured by flow cytometry. In fig. 1.A, the differences between strains are extremely significant ($p<0.0001$). Statistical analysis was performed using a two-way ANOVA test.

HSP12 deletion strain displays an increase in intracellular trehalose content

Trehalose is widely recognized as one of the most effective compounds in the protection of cellular structures against the damage caused by freezing. The accumulation of trehalose in fungi is associated in general with periods of starvation and reduced growth rate (Kuenzi & Fiechter, 1972; Lillie & Pringle, 1980; Panek, 1975). For our freezing experiments, cells were harvested in stationary-phase, where, trehalose synthesis is particularly intensive (Kuenzi & Fiechter, 1972; Lillie & Pringle, 1980; Panek, 1975). In addition, it has been previously described that another HSP disrupted strain, Δ*hsp104,* displayed increased intracellular trehalose concentrations (Fujita *et al.*, 1998). In order to ascertain if Δ*hsp12* mutant, similarly to Δ*hsp104,* could have an increase in intracellular trehalose concentration, which could be masking any freezing sensitivity caused by *HSP12* deletion, we assessed trehalose content of this strain. The results showed that when compared to the wild type strain, Δ*hsp12* mutant presented a 50% higher intracellular trehalose concentration (table II).

Table II. Intracellular trehalose concentrtion in the strains under study

Strains	[trehalose]IN (mg/g cells)
wild type	35.3 ± 3.4
Δ*hsp12*	54.2 ± 5.6
Δ*tps1* -p_{HSP12}	5.4 ± 0.6
Δ*tps1* -p_{empty}	7.71 ± 0.6

We further confirmed that this increase in trehalose content was specific for the disruption of *HSP12* gene, since no differences in the trehalose content were observed for the same wt strain (BY4742) disrupted in another gene, Δ*dur1,2* (results not shown). The results supported our hypothesis that an increase in intracellular trehalose concentration could be responsible for the higher freezing resistance observed, not allowing to differentiate the possible effects of *HSP12* deletion. In addition, they suggest that under these circumstances trehalose may in fact be more critical for survival than Hsp12p likewise reported by some authors (Hottiger *et al.*, 1989; Kandror *et al.*, 2002), or at least may replace for its function.

Hsp12p contributes to the yeast freezing resistance

We next wanted to check whether the overexpression of *HSP12* might also have an effect in freezing tolerance. The fact that HSPs and trehalose both contribute to yeast stress tolerance (Iwahashi *et al.*, 1997; Sales *et al.*, 2000) is an obstacle to the assessment of their individual role in the acquisition of freezing tolerance by cells. To be able to evaluate any effect of *HSP12* overexpression, minimizing trehalose influence, we used a trehalose-6-phosphate synthase *(TPS1)* null mutant, which is responsible for the first step in trehalose synthesis. Mutations in *TPS1* gene are reported to result in lack of ability to synthesize trehalose. Nevertheless, since previous studies (Plourde-Owobi *et al.*, 1999; Plourde-Owobi *et al.*, 2000) and more recently Jules and co-workers (Jules *et al.*, 2008), reported that accumulation of trehalose may raise from the active uptake of exogenous trehalose (that comes from the 'yeast extract' used to make the culture medium, 29) by the *AGT1*-encoded transporter (De Hertogh *et al.*, 2006), we also determined trehalose content of the transformed Δ*tps1*–p_{empty} *and* Δ*tps1*–p_{HSP12} strains. Intracelular trehalose content was residual and similar in both strains (7.71 and 5.4 mg/g cells respectively, table II), and therefore allowed to effectively reduce strain's background from trehalose.

The expression of *HSP12* in this strain was confirmed by real time RT-PCR. As can be seen in fig. 3B, Δ*tps1*–p_{HSP12} strain showed a significant increase in expression of *HSP12* gene when compared with Δ*tps1*–p_{empty} strain.

We also assessed proper synthesis of Hsp12 protein by SDS-PAGE. For that cells of Δ*hsp12* were transformed with plasmid pRSHSP12 and with control vector. When we analyzed Δ*hsp12* strain transformed the pRSHSP12, it was possible to confirm the presence of a band corresponding to Hsp12p, which was absent in Δ*hsp12* strain untransformed or transformed with the empty vector (fig. 2). This was the same band that disappeared from Δ*hsp12* strain when compared with the wild type. The results indicated that the plasmid was functional in the Δ*hsp12*-p_{HSP12} strain (fig. 2).

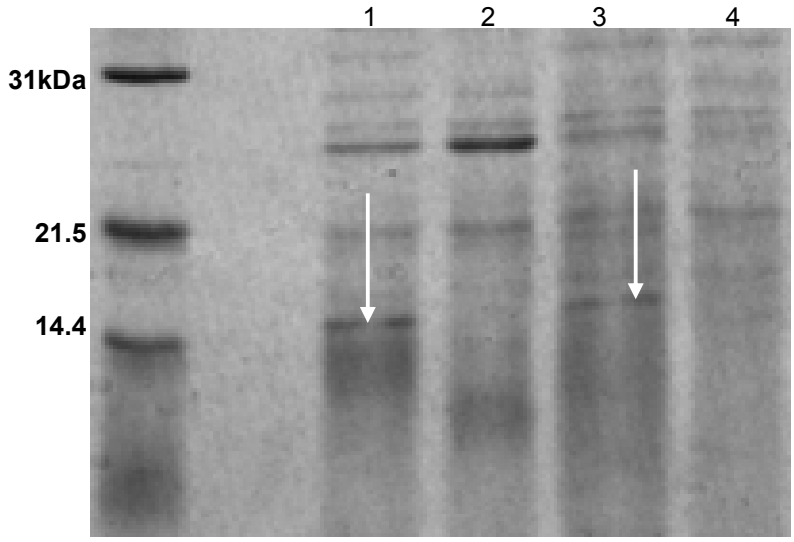

Fig. 2. Detection of Hsp12p in SDS-PAGE gel, in the strains under study. Total proteins were extracted from yeast in 50 mM NaCl 10 mM Tris-HCL pH 7.5 and insoluble proteins were precipitated after heating the total extract in the same buffer at 80 ºC for 10 min; heat-coagulated proteins were removed by centrifugation. The migration of the molecular weight marker (M) of known size (KDa) is denoted on right hand side of the gel. The arrow shows the electrophoretic migration of the protein Hsp12p (15 kDa) as already described in (Praekelt & Meacock, 1990; Stone *et al.*, 1990). The 15 kDa protein is absent on lane corresponding to Δ*hsp12* and Δ*hsp12*-$p_{empty.}$
Lane 1 - wt
Lane 2 - Δ*hsp12*
Lane 3 - Δ*hsp12*–p_{HSP12}
Lane 4 - Δ*hsp12*– p_{empty}

Stationary phase cell of Δ*tps1*– p_{empty} and Δ*tps1*–p_{HSP12} strains were frozen at -20 ºC and viability was determined along time. Δ*tps1* strain overexpressing *HSP12* showed an increase in resistance to freezing when compared to the strain harbouring the empty vector (fig. 3A).

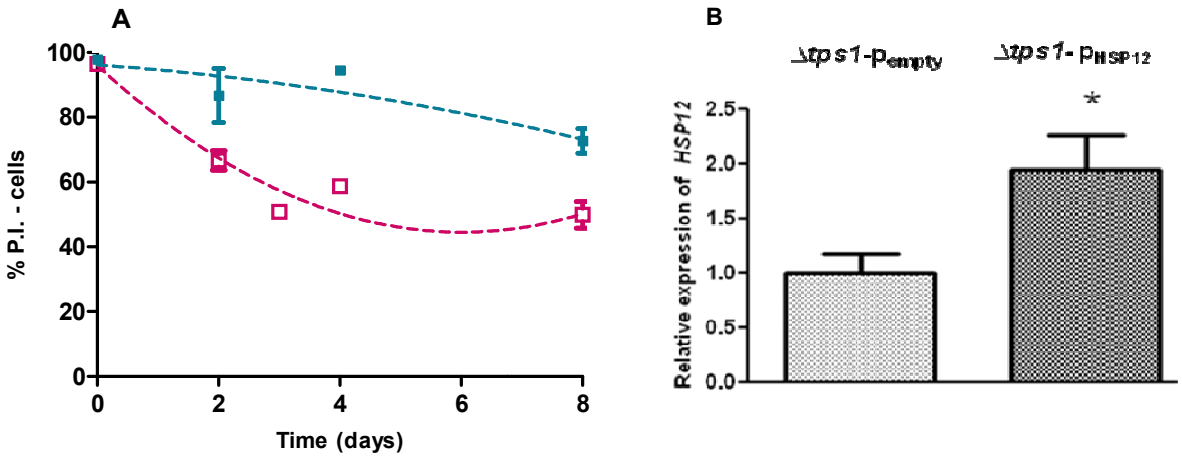

Fig. 3. Cell viability analysis of the Δ*tps1* transformed strains, after freezing at -20 °C. Cells of Δ*tps1* strain transformed with the plasmids pRS41H (□) and pRSHSP12 (■) were grown in YPD supplemented with 300 mg/L hygromycin at 30 °C until stationary phase (OD_{640nm} ≈ 3-4), and then subjected to prolonged storage at -20 °C. **A**) Viability of the cells along freezing was determined by propidium iodide exclusion as measured by flow cytometry. Two-way ANOVA analysis revealed that differences between strains were very significant (p=0.0032). **B**) Expression analysis of *HSP12* gene by real time RT- PCR in the Δ*tps1* transformed strains. Values of R.E. were calibrated with respect to the Δ*TPS1* strain harboring the empty vector, with a R.E. value set to 1.

Heat stress tolerance is also increased in HSP12 overexpressing strain

Previous work has indicated that mutations in *HSP12* gene did not cause a detectable phenotype under several stress conditions, namely heat stress (Praekelt & Meacock, 1990; Varela *et al.*, 1995). To investigate if overexpression of *HSP12* gene could also influence heat shock response in these trehalose-6-phosphate synthase null mutant, we analyzed viability of Δ*tps1*–p_{empty} and Δ*tps1*–p_{HSP12} strains in response to a temperature shift from 25 to 50 ºC. When we assessed this system with reduced intracellular trehalose, we observed that the strain overexpressing *HSP12* showed an increased resistance to heat shock stress when compared with the strain harbouring the empty vector (fig. 4), sustaining the fact that Hsp12p effectively contributes to heat shock resistance.

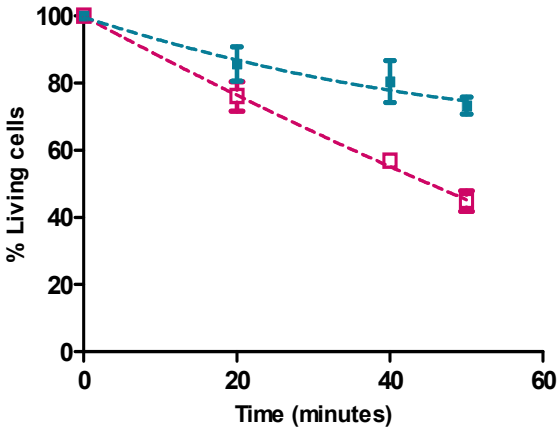

Fig. 4. Heat shock tolerance at 50 ºC of Δtps1 transformed strains**.**
Effect of heat-shock on cell viability of Δ*tps1* strains transformed with the empty plasmid (□) and *HSP12* expressing plasmid (■). The cells of both strains were grown in YPD supplemented with 300 µg L^{-1} hygromycin at 25 °C until stationary phase (OD_{640nm} ≈ 3-4). Cells were then diluted to (OD_{640nm} = 1) and subjected to a temperature shift from 25 to 50 ºC. Viability of the cells was determined by C.F.U., at the indicated times. Two-way ANOVA analysis revealed that differences between strains were extremely significant (p<0.0001).

Increase of intracellular trehalose content in Δ*hsp12* strain is not due to increase expression of *TPS1*

Subsequently, we addressed if *HSP12* could directly affect *TPS1* expression, which could be responsible for the observed differences in the intracellular trehalose levels. In order to determine if increase in trehalose content in Δ*hsp12* strain was due to an induction of *TPS1* expression, we measured *TPS1* mRNA levels by real time RT-PCR in wt and Δ*hsp12* strains. The data showed no significant differences in the expression of *TPS1* in the two strains (fig. 5).

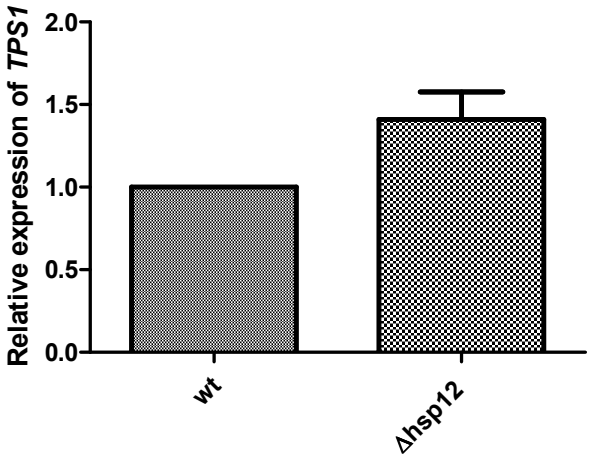

Fig. 5. Real-time RT-PCR measuring expression of *TPS1.* Expression was measured in wt and Δ*hsp12.* Differences between wt and Δ*hsp12* strains were not significant (*P* = 0.13). Comparisons between strains were performed employing an unpaired T-test.

HSP12 deletion does not affect trehalose mobilization but reduces the heat-induced Increase of intracellular trehalose concentration

The higher trehalose concentration in the Δ*hsp12* strain could also result from a decreased removal by trehalase, as found for Δ*hsp104* strain (Iwahashi *et al.*, 1998). We next tried to evaluate if *HSP12* deletion could originate defects in trehalose mobilization upon different stimuli. For that purpose, we examined the intracellular trehalose concentration in stationary phase cells of both strains, after a glucose pulse or a temperature shift to 50 °C. Trehalase is activated under both conditions, originating a rapid decrease of intracellular trehalose concentration (Carrillo, 1992; Thevelein, 1984; Thevelein, 1991). The results showed that there were no significant differences in trehalose degradation over time, either after a glucose pulse or during incubation at 50 °C (fig. 6).

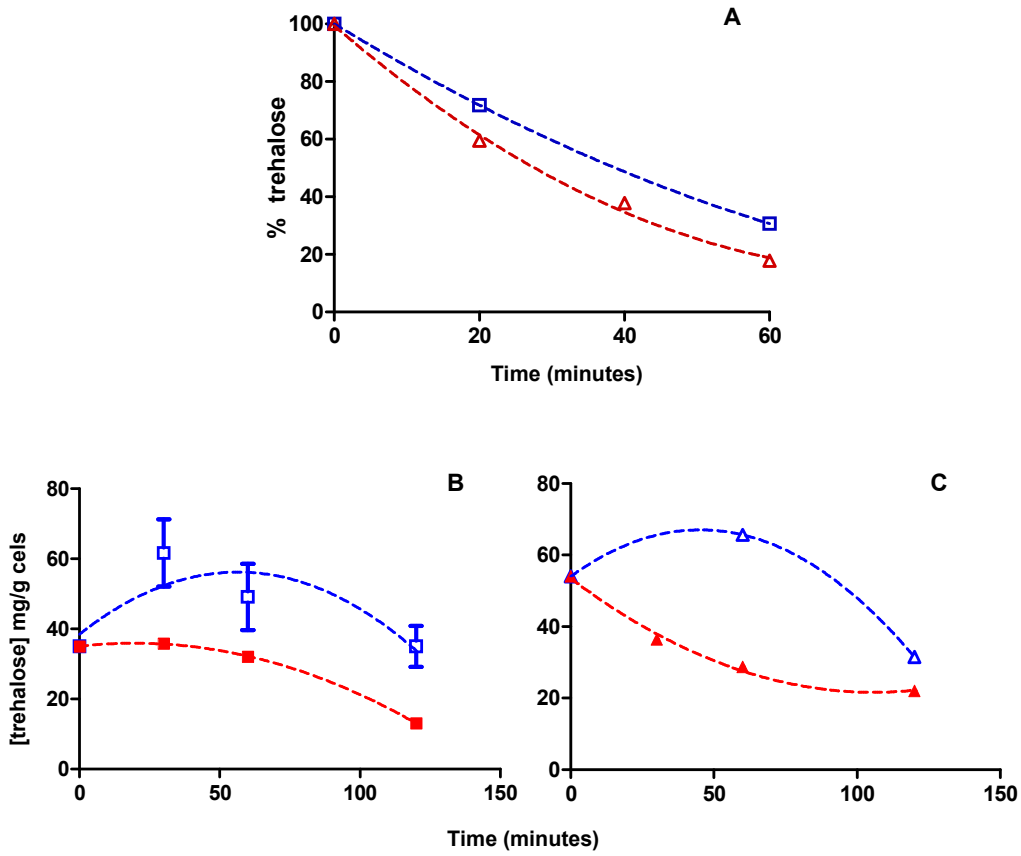

Fig. 6. Intracellular trehalose concentration, over time in stationary phase cells:
- After a glucose pulse (A). Yeast cells of BY4742 (□) and Δ*hsp12* (△) strains were grown in YPD at 30 °C until stationary phase, and transferred to fresh YPD medium (glucose pulse). Initial values of intracellular trehalose concentration were: [trehalose] $_{BY4742}$= 35 mg/g cells and [trehalose] $_{\Delta hsp12}$ = 54 mg/g cells. Samples were taken at the indicated times, and trehalose was extracted as described previously in "methods". A representative experiment is shown; experiments were repeated three times with similar results;
- After heat shock at 50 ºC in the wild type strain (B) or in Δ*hsp12* strain (C). Cells were grown in YPD until stationary phase and exposed to a temperature shift from 25 to 50 ºC. Open symbols are for the control condition at 25 ºC, and filled symbols are for the 50 ºC experiment.

To determine if deletion of *HSP12* would also increase trehalose accumulation during a mild heat treatment, we grew wt and Δ*hsp12* cells at 25 °C and then subjected them to incubation at 37 °C. As shown in fig. 7, the temperature shift from 25 to 37 ºC caused a rise of about fourfold in wild type strain in accordance with previous research described (Parrou *et al.*, 1997). On the contrary, Δ*hsp12* strain displayed only a one and a half fold increase in trehalose content. The results show that contrary to what happens upon entrance into stationary phase, where the trehalose accumulation is increased, Δ*hsp12* strain shows a decreased capacity to accumulate trehalose upon a mild heat stress.

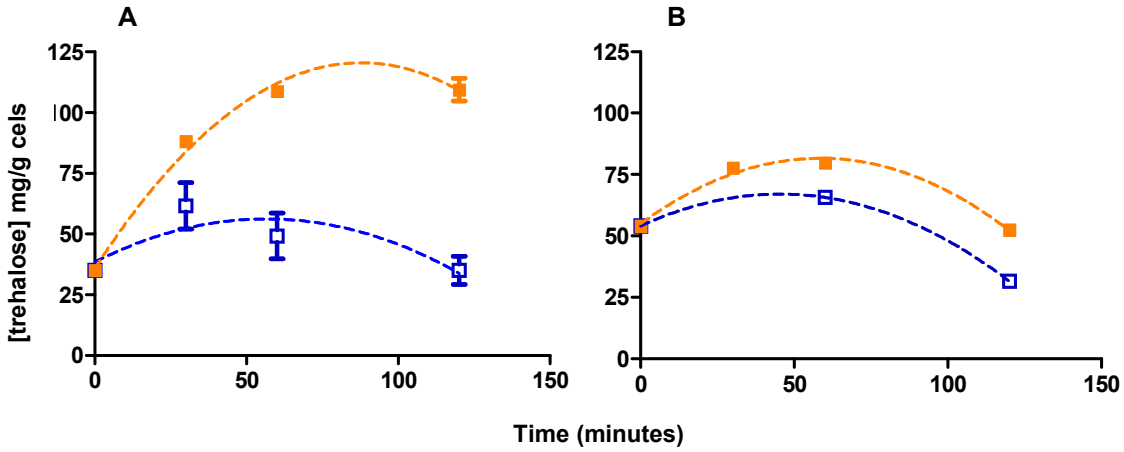

Fig. 7. Effect of heat shock at 37 ºC on trehalose levels in the wild type and Δ*hsp12* strains**.** wt (**A**) and Δ*hsp12* (**B**) cells were grown in YPD at 25 °C until stationary phase and exposed to a temperature shift from 25 to 37 ºC. Open symbols are for the control conditions at 25 ºC, and filled symbols are for the 37 ºC experiments

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Discussion

In this paper we report that disruption of the cold stress-inducible *HSP12* gene does not result in increased sensitivity to freezing storage. Our results point to a redundant role of Hsp12p under freezing conditions and identify trehalose as the cell component that is able to replace its functions under such conditions. In fact, when the intracellular trehalose content was decreased to residual levels by *TPS1* deletion, an increase in freezing resistance was observed after *HSP12* overexpression. Hsp12p has been described to localize both at the cytosol, the plasma membrane (Sales *et al.*, 2000), and extracellularly at the cell wall (Motshwene *et al.*, 2004). Using a model liposome system, it was also shown that Hsp12p acts in a similar way to trehalose in the protection of membrane integrity against desiccation (Sales *et al.*, 2000). Taking into account that loss of cell viability under freezing conditions depends directly on the yeast capacity to preserve its membrane integrity (Alves-Araújo *et al.*, 2004a) the results suggest that the protection role of Hsp12p during freezing storage is most probably exerted at the plasma membrane level.

Using the Δ*tps1*-p_{HSP12} strain we could also show an increase in heat shock resistance by *HSP12* overexpression, demonstrating that Hsp12p can also, in fact contribute to heat resistance. Deletion of *HSP12* led to an increase in intracellular trehalose content, which was not accompanied by a significant induction of the *TPS1* gene, or by a decreased capacity in trehalose mobilization. Our results support the interpretation that such increase may result from activation of trehalose-6-phosphate synthase. This is unlike to what was found for *HSP104* deletion, where the observed increase of trehalose content was attributed to a decrease in both neutral trehalase and trehalose-6-phosphate synthase activities (Iwahashi *et al.*, 1998).

Although absence of *HSP12* led to higher induction of trehalose accumulation upon entry into stationary phase, it decreased the ability of the yeast cells to accumulate high levels of trehalose in response to stress by a mild heat shock. These results suggest that Hsp12p may have a role in the induction of trehalose accumulation in response to heat shock.

In conclusion, by overexpressing *HSP12* in a Δ*tps1* strain, we could show that Hsp12p contributes to both freezing and heat resistance, and that its protective role during freezing storage is interchangeable with that of trehalose. Moreover, the developed Δ*tps1*-pHSP12 system can still prove to be useful to assess the individual contribution of Hsp12p in other conditions where it shares roles with trehalose.

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Chapter 7

General Discussion

Concluding remarks

The biotechnological interest in *Torulaspora delbrueckii* has increased in recent years due to its particularly high freezing and osmotic tolerance (Almeida & Pais, 1996a; Alves-Araújo *et al.*, 2004a; Hernandez-Lopez *et al.*, 2003). These features made this organism a candidate of potential value for the baking industry. However, knowledge on this yeast is still far from the vast knowledge on the traditional baker's yeast *Saccharomyces cerevisiae*. Therefore, we centered our main attention on an investigation to gain insight into the physiology and biochemistry of *T. delbrueckii.*

In modern food technology traceability is a crucial request, so to begin with, we needed to establish a hasten method to discriminate among *T. delbrueckii* strains. This technique would enable the correct identification of the inoculated strain from the remaining yeast flora present in the bread dought. In the last years, several methodologies of typing based on DNA polymorphisms have been developed which allowed the discrimination among closely related yeast strains. In the present work two different genetic fingerprinting techniques (karyotypes analysis and mtDNA restriction analysis) were used for the detailed genotyping of *T. delbrueckii* strains. The analysis of the polymorphisms produced by each of the methods allowed distinguishing *T. delbrueckii* at the strain level. Mitochondrial DNA restriction analysis proved to be a good technique to differentiate among *T. delbrueckii* strains isolated from the same ecosystem even when they are genetically very closely related. Chromosome separation by pulsed-field electrophoresis revealed considerable variability in the chromosomal constitution of the strains studied, and also turned out to be a useful method to discriminate among *T. delbrueckii* strains. However this method of chromosome karyotyping may be too complex, laborious and time-consuming for the analysis of numerous yeast isolates in contrast with mtDNA restriction analysis. We suggest that mtDNA restriction analysis is a convenient instrument for quick identification of *T. delbrueckii* strains.

While two of the most important traits for large-scale bakers yeast production are growth rate and biomass yield on sucrose, leavening ability depends mainly on fermentative capacity of maltose. In addition, the pattern of sugar utilization and regulation also determines the yeast capacity to rapidly adapt when changing from the sucrose-rich growth medium to the dough. In this work we have undertaken physiological and biochemical studies of *T. delbrueckii* in batch cultures with sugars present in molasses and in bread dough, using them alone and in mixtures. The results indicated that *T. delbruecki* behaves very similarly to *S. cerevisiae* with respect to sugar utilization and regulation patterns. We also show a more efficient modulation of the respiratory metabolism in *T. delbrueckii* under aerobic conditions, which represents an asset for the large-scale production of the yeast. Furthermore, the comparative analysis of specific sugar consumption rates and transport capacities suggested that the transport step limits both glucose and maltose metabolism.

So far, just one glucose transporter had been identified in *T. delbrueckii*, the low-affinity glucose transporter *LGT1* (Alves-Araújo *et al.*, 2005). Southern blots analysis of this yeast genome revealed the existence of several genes with high homology to *LGT1* gene, suggesting the existence

of several hexose transporters in *T. delbrueckii.* Consistent with this result, was the difficulty we came across when trying to disrupt *LGT1* gene. Δ*lgt1* null mutant did not present any growth defect in glucose media, probably due to existence of other physiologically relevant hexose transporters in this yeast that compensate the loss of Lgt1p. Also in agreement with these findings, was the small decrease of glucose uptake rates upon the disruption of this transporter gene. The occurrence of several hexose transporters had been first suggested by the isolation, from a genomic library of this strain, of several plasmids that could complement the glucose growth defect of the *S. cerevisiae* hexose transport-null mutant (Alves-Araújo *et al*., 2005). An in-depth study of one of these plasmid resulted in the isolation and characterization of a second glucose transporter, *IGT1*, in *T. delbrueckii*. Functional characterization of Igt1p in *S. cerevisiae hxt*-null strain showed that it encodes an intermediate-affinity transporter able to mediate the uptake of glucose, fructose and mannose. Furthermore, apparent K_m of Igt1 transporter could be modulated by medium glucose concentration as described for *S. cerevisiae* Hxt2p (Reifenberger *et al*., 1997). Cells of *S. cerevisiae hxt*-null strain transformed with *IGT1,* when grown in 0.1 % glucose displayed biphasic uptake kinetics with an intermediate- (K_m = 6.5 ± 2.0 mM) and a high-affinity (K_m= 0.10 ± 0.01 mM) component. However it is probable that *T. delbrueckii* has other high affinity transporters further than Igt1p. The existence of other high affinity transporters is supported by the kinetics of glucose transport in *T. delbrueckii* cells grown in 2% glucose, which displays a mediated glucose transport activity best fitted assuming a biphasic Michaelis–Menten kinetics with a low- and a high affinity component, not displayed by Igt1p when cells are grown at this glucose concentration.

Though the phylogenetic closeness of *T. delbrueckii* and *S. cerevisiae*, the differences observed between the two species, show that the behavior or even the methods to apply to the former yeast not always can be inferred from those of *S. cerevisiae.* For instance when we tried to disrupt *T. delbrueckii LGT1* gene the current methods used for *S. cerevisiae* were not suitable, and an optimized disruption method had to be developed. On the other hand, *S. cerevisiae* is a widely used organism in both fundamental and applied research and a vast knowledge about the regulation of its genes and metabolic pathways is available, making it an ideal model for studies on stress tolerance.

Cells of *S. cerevisiae* exhibit a stress response when exposed to severe environmental conditions. They respond to such stress by rearranging certain metabolic pathways to synthesize proteins and compounds, which help protect the cellular environment against damage and thus counteract the stress. In *S. cerevisiae*, one of these proteins is the small hydrophilic stress response protein Hsp12p, the synthesis of which is markedly upregulated by various forms of stress (Karreman & Lindsey, 2005; Motshwene *et al*., 2004; Mtwisha *et al*., 1998; Pedruzzi *et al*., 2000; Praekelt & Meacock, 1990; Sales *et al*., 2000; Varela *et al*., 1995). We have investigated the effects of deleting the *HSP12* gene on the response of yeast to freezing storage at -20 ºC. Our data revealed that *HSP12* deletion does not result in increased sensitivity to freezing storage. Interestingly, the response of the yeast cell to the loss of *HSP12* gene was to increase the trehalose concentration, with the surprising result that Δ*hsp12* cells were more freeze tolerant than the wild-type cells. Recently (Shamrock & Lindsey, 2008) also described that the increase on intracellular trehalose levels in the Δ*hsp12* strain increased tolerance to desiccation

and to exogenously applied H_2O_2.

This correlation between increased trehalose levels and an increase in freezing resistance is only found on stationary phase yeast cells. Thus, mid-exponential cells of both wild type and Δ*hsp12* exhibited no differences in freezing resistance. These data is in agreement with the fact that synthesis of Hsp12 is repressed during exponential phase. As a all, the results suggest that trehalose rather than Hsp12p is the main factor in countering freezing stress in cells growing under normal stress conditions.

Trehalose synthesis and *HSP12* transcription are both increased by many of the same stress conditions and both enable similar protective functions, suggesting that they may have synergistic roles. It has previously been proposed that a small protein or peptide, such as a hydrophilin, may function in a similar manner to trehalose during desiccation (Singh *et al.*, 2005). One place where the functions of Hsp12p and trehalose overlap is at the plasma membrane, where both molecules have been shown to preserve the structural and functional integrity (Sales *et al.*, 2000; Sharma, 1997). We would therefore postulate that the increased synthesis of trehalose in response to *HSP12* deletion was applied as a protectant against freezing. However, it would appear that Hsp12p itself imparts protection against freezing at -20 ºC and heat shock, since Δ*tps1* cells transformed with *HSP12*, showed a clear increased in the resistance to these stresses.

Shamrock *et al*. proposed that Hsp12p role at the plasma membrane would be to modulate the fluidity of the membrane by interacting with the phospholipid head groups, so the lack of Hsp12p in the Δ*hsp12* strain would result in a more rigid membrane (Shamrock & Lindsey, 2008). This effect would trigger the up-regulation of the HOG (high osmolarity glycerol) pathway leading to an increase in the trehalose content, since *TPS1* gene, which codes for trehalose-6-phosphate synthetase, is a target of this pathway. However, we did not observe an increase in TPS1 mRNA in Δ*hsp12* strain, the results rather suggesting that trehalose increase could originate from the post-transcriptional activation of trehalose-6-phosphate synthetase.

Our results also suggest that Hsp12p may have a role in the induction of trehalose accumulation in response to heat shock, as lack of this protein impaired full increase in intercellular trehalose upon a mild heat stress.

Future perspectives

Nowadays yeast strains used in bread industry are involved in large-scale processes and hence are exposed to more extreme stresses. On the other hand, development of new products and more versatile processes also require yeast strains with new traits. This work aimed to solve some of those emergent problems/needs in the bread-making industries, selecting, characterizing and constructing yeast strains resistant to freezing, and strains with important qualities for application in baking industry.

Despite the accomplishments reported in this thesis, many important questions remain to be answered regarding sugar transports and freezing resistance in *T. delbrueckii*. How many hexose transporters are present in *T. delbrueckii*? What are their affinities and regulation? Is it *T. delbrueckii* similar to *K. lactis* as speculated by Alves-Araújo (Alves-Araújo *et al*., 2005), based on comparison sequencing data and regulatory studies of *LGT1* expression? Or is this yeast more comparable to *S. cerevisiae* as it might be suggested by their resemblance in sugar utilizations patters (Alves-Araújo *et al*., 2007) or even by the Igt1p similarities with *S. cerevisiae* Hxt2p? Regarding freezing tolerance, what are the transcriptional changes in response to *hsp12* deletion? How does it affect stress signalling pathways? Is there an Hsp12p homologous in *T. delbrueckii*? If so, could it be associated with its higher capacity to preserve plasma membrane integrity?

It is clear that answer to those questions may only come from future studies. Hereafter we will list some additional research that may contribute to clarify some of these issues.

We have established a convenient method to quickly differentiate *T. delbrueckii* strains which will assist in the exploitation of our baker´s yeast collection isolated from homemade bread doughs (Almeida & Pais, 1996b), searching for strains with potential applications at the baking industry. In parallel, we will also continue to study the genetic variability within *T. delbrueckii* strains and the genetic features that are responsible for the observed biodiversity.

Evidently, we are particularly interested to continue the characterization of the two strains, *T. delbrueckii* PYCC 5321 and PYCC 5323, since their biotechnological potential is already established (Alves-Araújo *et al*., 2004a; Alves-Araújo *et al*., 2007; Hernandez-Lopez *et al*., 2003). We will carry on the studies on sugar transport in these strains. Some aspects on *IGT1* regulation remain to be elucidated. We intend to study the influence of both disruption and overexpression of *T. delbrueckii* PYCC 5321 *IGT1* gene, in its fermentation ability. It would be interesting to construct a double *IGT1* and *LGT1* mutant to assess the contribution of both transporters to this yeast fermentation capacity.

Since both *LGT1* and *IGT1* display high similarity with Hxt9p/Hxt11p (75% of identity), two genes not regulated by glucose, which play a role in drug resistance (Nourani *et al*., 1997) it would be appealing to assess the role of these genes in drug tolerance.

HSP12 expression is controlled by several stress signaling pathways, integrating signals from both general stress response, *HSF1*, and HOG pathway (Aguilera *et al*., 2007; Varela *et al*., 1995). It would be interesting to test the effect of *hsp12* deletion in strains affected in the different pathways. Since *TPS1* and *HSP12* share these regulation pathways monitoring the effects on trehalose accumulation could help to unravel how balance between Hsp12 and trehalose is regulated.

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Chapter 8

Appendix

Appendix índex

Appendix I - DNA

Restriction enzyme(s) digestion of plasmid minipreps

Adapted from (Sambrook J, 1989).

Add 2 µl of appropriate restriction enzyme buffer (10x), 11.5 (or 11) µl of ultra pure water, 0.5 µl restriction enzyme A (generally Roche 10 U/µl), (0.5 µl restriction enzyme B (generally Roche 10 U/µl) for double digestions) and 6 µl of plasmid DNA miniprep or midiprep. Incubate at appropriate temperature (37 ºC for most enzymes) for at least 3 hours. Analyse digestion fragments by gel electrophoresis.

Standard ligation of DNA fragments to plasmid vectores

Adapted from (Sambrook J, 1989).

Digest DNA fragment and plasmid vector with appropriate restriction enzyme(s). Purify using QIAquick Gel Extraction Kit (QIAGEN).

To 50 ng of digested plasmid vector add digested DNA fragment enough to get a molar vector:insert ratio of 1:5 (insert quantity (ng) = (insert size x 50 x 5)/ plasmid size). Add 1 µl ligase buffer (10x), 1 µl of ligase T4 1 U/ µl (Roche) and, if necessary, ultra pure water to a final volume of 10 µl. Incubate overnight at 4 ºC.

Preparation and transformation of competent E. coli (XL1 Blue) SEM method

Adapted from (Inoue *et al.*, 1990).

Reagents

DMSO (Sigma)

IPTG (isopropyl-β-D-thiogalactopyranoside (Sigma)

40 mg/ml in sterilized destilled water

Luria-Bertani (LB)-Ampicillin agar

Tryptone	10 g/l
Yeast extract	5 g/l

NaCl 10 g/l

Agar 2%

Autoclave, cool to 50 ºC and add a stock solution of ampicillin 100 mg/ml to a final concentration of 75 mg/l, pour onto Petri plates.

LB medium (liquid)

Tryptone 10 g/l

Yeast extract 5 g/l

NaCl 10 g/l

Autoclave

SOB

Tryptone 2%

Yeast extract 0.5%

NaCl 10%

Autoclave, add sterilized $MgCl_2$ to a final concentration of 20 mM.

TE buffer

PIPES 10 mM

$CaCl_2$ 5 mM

KCl 250 mM

Dissolve, adjust pH to 6.7 with KOH and add $MnCl_2$ to a final concentration of 55 mM, sterilize the solution by filtration and keep at 4 ºC.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

40 mg/ml in dimethylformamide (DMF)

Preparation of competent *E. coli* (XL1 Blue)

Inoculate 250 ml SOB medium in a 2 L flask with 10 colonies;

Grow at 18 ºC with vigorous shaking (200-250 rpm) until an OD_{600} of 0.6.

Cool on ice for 10 min.

Spin cells down at 4ºC for 10 min at 2500 x g.

Ressuspend cells in 80 ml of ice-cold TB buffer.

Cool on ice for 10 min.

Spin cells down at 4 ºC for 10 min at 2500 x g.

Gently ressuspend pellet in 20 ml of ice-cold TB and add DMSO to a final concentration of 7 %.

Leave on ice for 10 min.

Distribute into 200 ml aliquots (into sterile, ice-cold 1.5 ml eppendorf tubes) and freeze in liquid nitrogen.

Store at -80 ºC.

Transformation of competent *E. coli* (XL1 Blue)

Thaw the competent *E. coli* cells on ice.

Add the experimental DNA (generally 1 µl of midi or mini and 10 µl of a ligation reaction) to 200 µl of competent cells.

Mix gently and incubate on ice for 30 min.

Heat-shock the tubes in a thermoblock, at 42 ºC for 30 s with gentle agitation.

Incubate the tubes on ice for 10 min.

Add 800 µl of SOC medium and incubate the tubes for at 37 ºC for 1 hour with vigorous shaking.

Spin cells down for a few seconds and discard about 950 µl of supernatant.

Ressuspend the pellet in the remaining 50 ul supernatant and plate on LB selective agar

plates. For blue-white colour screening add X-Gal 40 mg/ml to a final concentration of 40 µg/ml and IPTG 40 mg/ml to a final concentration of 40 µg/ml. Allow the plates to dry before plating the transformation mixtures.

Incubate the plates at 37 ºC overnight.

Preparation of plamid DNA (Miniprep)

Adapted from (Sambrook J, 1989)

Reagents

Alkaline lysis solution I

0.2 M NaOH

1% (w/v) SDS

Alkaline lysis solution II

NaAc 3 M, pH 5.2

Ethanol 100% and 70% (v/v)

Plate eight *E. coli* colonies, per Petri plate, on LB selective agar. Incubate the plates overnight at 37 ºC.

Resuspend ¾ of biomass, from each colony, in 200 µl of destilled water. Vortex for10 sec.

Add 200 µl of alkaline lysis solution I. Close the tubes tightly and mix the contents by inverting the tubes rapidly for four times. Do not vortex.

Add 200 µl of alkaline lysis solution II. Close the tubes tightly and disperse alkaline solution II through the viscous bacterial lysate by inverting the tubes rapidly for four times.

Centrifuge the bacterial lysate at maximum speed for 5 min at 4 ºC. Transfer the supernatant to a clean tube.

Precipitate nucleic acids from the supernatant by adding 500 µl of ethanol. Mix the solution by inverting four times.

Collect the precipitated nucleic acids by centrifugation at maximum speed for 10 min at

4 ºC.

Remove the supernatant and add 500 µl of 70 % ethanol to the pellet. Centrifuge at maximum speed for 5 min at 4 ºC.

Remove all of the supernatant and store the open tubes at room temperature until all the ethanol has evaporated.

Dissolve the nucleic acids in 30 µl of TE-RNase and incubate for 1 h at 37 ºC for RNA digestion.

Store nucleic acids at -20 ºC.

Spectrophotometric estimation of DNA purity and quantitation

As described in “Molecular Cloning” (Sambrook J, 1989), it is possible to quantify nucleic acids and to evaluate their purity by spectrophotometric analysis. DNA and RNA absorb light of 260 nm wavelength, proteins (aromatic amino acids) absorb light of 260 nm wavelength too, but absorption is much stronger at 280 nm. The ratio A_{260}/A_{280} gives an estimation of DNA purity. For pure DNA, the A_{260}/A_{280} ratio is about 1.8. Spectrophotometric conversion: 1 A_{260} of double-stranded DNA = 50 mg/ml.

1 A_{260} of single-stranded DNA = 33 mg/ml

1 A_{260} of single-stranded RNA = 40 µg/ml

Enzymatic treatment of DNA

Restriction enzyme digestion was carried out according to standard procedures described by (Sambrook J, 1989). Depending on the enzymes used and their cutting sites, stickyended (5’- or 3’-protruding single strand DNA) or blunt-ended DNA fragments were generated. Restricted DNA fragments were purified either by gel electrophoresis and extraction using a QIAGEN gel extraction kit (Hilden, Germany), or by using a QIAGEN PCR and nucleotide purification kit (Hilden, Germany). DNA fragments with compatible cohesive ends were ligated using T4 DNA ligase which

S. cerevisiae transformation (LiAc conventional method)

Adapted from (Gietz *et al.*, 1995).

Reagents

1M lithium acetate (LiAc) stock solution

Prepare in bidistilled water, sterilize by filtration.

polyethyleneglycol 3500 (w/v)

Prepare in bidistilled water, sterilize b filtration or in the autoclave and store in a securely capped bottle.

Single-stranded carrier DNA (Deoxyribonucleic acid sodium salt from Salmon testes, Sigma) 2 mg/ml In sterile TE pH B mix vigorously and store aliquots al-20 ºC .

Day1

Inoculate the yeast strain into 5 ml of liquid medium (2x YPD or SC selection medium) and incubate overnight on a rotary shaker at 200 rpm and 30°C.

Day2

1. Determine the titer of the yeast culture by pipetting 10 ml of cells into 1.0 ml of water in a spectrophotometer cuvette and measuring the OD at 600 nm. For many yeast strains a suspension containing 1 x 10^6 cells/ml will give an OD600 of 0.1. Alternatively, titer the culture using a hemocytometer. see note:

Note:

i) Dilute overnight YPD or SC cultures 10^{-1} or more in water.

ii) Carefully place 10 µl of the cell suspension between the cover slip and the base of haemocytometer. Let the cells settle onto the haemocytometer grid for a few minutes. The grid area is typically 1 square millimeter, divided into 25 equal-sized squares, and the volume measured is 10^{-4} ml.

ii) Count the number of cells in 5 diagonal squares

iv) Calculate the cell titer as follows: cells counted x 5 x dilution factor x

1/volume measured by the 25 squares of the haemocytometer. 239 cells x 5 x 10 (dilution factor) x $1/10^{-4}$ml = 1.2 x 10^8 cells/ml.

v) ***Saccharomyces cerevisiae*** divides by budding from a mother cell. Count budded cells as a single cells. Count cells with equal bud sizes as two cells when there is evidence of additional buds forming on either cell. Some strains form clumps of cells which reduce plating efficiency. A single clump of cells will only give rise to one colony on a plate, which may complicate further analysis.

2. Transfer 50 ml of 2x YPD and add 2.5 x 10^8 cells to give 5 x 10^6 cells/ml.

3. Incubate the flask on a rotary or reciprocating shaker at 30°C and 200 rpm.

Note:

i) It is **very important** to allow the cells to complete **at least two divisions**.

ii) This will take 3 to 5 hours (depending on the yeast species).

iii) This culture will give sufficient cells for 10 transformations.
iv) Transformation efficiency (transformants/ µg plasmid/10^8 cells) remains constant for 3 to 4 cell divisions.

4. When the cell titer is at least 2 x 10^7 cells/ml, which should take about 4 hours, harvest the cells by centrifugation at 3000 *g* for 5 min, wash the cells in 25 ml of sterile water and ressuspend in 1 ml of sterile water.

5. Boil a 1.0 ml sample of carrier DNA for 5 min and chill in an ice/water bath while harvesting the cells.

It is not necessary or desirable to boil the carrier DNA every time. Keep a small aliquot in your own freezer box and boil after 3-4 freeze-thaws. But keep on ice when out.

6. Transfer the cell suspension to a 1.5 ml microcentrifuge tube, centrifuge for 30 sec and discard the supernatant.

7. Add water to a final volume of 1.0 ml and vortex mix vigorously to resuspend the cells.

> **Note:**If the cell titer of the culture is greater than 2 x 10^7 cells/ml the volume then increase the volume to maintain the titer of this suspension at 2 x 10^9 cells/ml. If the titer of the culture is less than 2 x 10^7 cells/ml then decrease volume.

8. Pipette 100 µl samples (ca. 10^8 cells) into 1.5 ml microfuge tubes, one for each transformation, centrifuge at top speed for 30 sec and remove the supernatant.

9. Make up sufficient Transformation Mix for the planned number of transformations plus one extra. Keep the Transformation Mix in ice/water.

Number of Transformations		
Reagents	1	5 (6X)
PEG 3500 50% w/v	240 µl	1440 µl
LiAc 1.0 M	36 µl	216 µl
Boiled SS-carrier DNA	50 µl	300 µl
Plasmid DNA	10 µl	60 µl
Water	24 µl	144 µl
Total	360 µl	2160 µl

10. Add 360 µl of Transformation Mix to each transformation tube and resuspend the cells by vortex mixing vigorously.

11. Incubate the tubes in a 30°C water bath or thermoblock for 30 min.

Incubate the tubes in a 42°C water bath or thermoblock for 20 min.

> **Note:**The optimum time can vary for different yeast strains. Please test this if you need high efficiency from your transformations.

12. Microcentrifuge at top speed for 30 sec and remove the Transformation Mix with a micropipettor.

13. Pipette 1.0 ml of sterile water into each tube; stir the pellet by with a micropipette tip and vortex .

> **Note:** Be a gentle as possible at this step if high efficiency is important. Excessive washing washes away transformants!!!!

14. Plate appropriate dilutions of the cell suspension onto SC or YPD selection medium. For transformation with need for high efficiency, do not plate cells immediately, instead add your cells to liquid selection medium (YPD + antibiotic or SC), and let them grow O.N. Proceed as described in 13.

> **Note:** When spreading yeast inoculum onto the plate gently distribute the fluid completely with a sterile glass rod with a minimum of strokes. Allow the fluid to be taken up by the plate prior to incubation.

15. Incubate the plates at 30°C for 3 to 4 days and count the number of transformants.

The transformation efficiency (transformants/1 µg plasmid/10^8 cells) can be determined by calculating the number of transformants in 1.0 ml of resuspended cells per 1.0 microgram plasmid per 10^8 cells. For example, if the transformation of 1.0 x 10^8 cells with 100 nanogram plasmid resulted in 500 colonies on a plate of SC dropout medium spread with 1 µl of suspension (usually dispensed into a 100µl puddle of sterile water on the plate).Transformation Efficiency = 500 x 1000 (plating factor) x 10 (plasmid factor) x 1 (cells/transformation x 10^8).Transformation Efficiency = 5 x 10^6 transformants/1.0 µg plasmid/10^8 cells. Transformation efficiency declines as plasmid concentration is increased (Gietz *et al.*, 1995) but the actual yield of transformants per transformation increases. For example, 100 nanogram of plasmid in a transformation might give a transformation efficiency of 5 x 10^6 and a yield of 5 x 10^5 transformants whereas with 1 µg of plasmid the Transformation Efficiency might be 2 x 10^6 and the yield 2 x 10^6 per transformation. In order to obtain 5 x 10^6 transformants it is simpler to set up two or three transformations with 1 µg of plasmid DNA, or a single 3 fold scaled up transformation, than to carry out 10 reactions with 100 ng of plasmid in each.

PCR amplification of DNA

Most PCR protocols were performed at the 25 µl -100 µl scale.

A typical 50 µl reaction mixture consisted of:

1 µl of 1-10 ng plasmid DNA or 50-100 ng genomic DNA

1 µl of 20 µM forward primer

1 µl of 20 µM reverse primer

1 µl of nucleotide mix (10 mM dNTP mix)

5 µl of PCR buffer without $MgCl_2$ (10x)

2 µl of $MgCl_2$ (50 mM)

0.25 µl of DNA polymerase (BioTAQ DNA Polymerase (5U/µl) from Bioline)

dH_2O

Colony PCR

PCR can be done directly from bacterial (or even yeast) colonies. Bacteria must be recovered with a toothpick from agar plates, and dissolved (or not in a drop of water.

Then boiled at maximum microwave temperature for 1 min (yeast require more time 2-3 min), and you may use it as template for the PCR reaction.

Appendix II - Protein

Tricine SDS-PAGE

Reagents:

AB MIX S 49,5% T; 3% C

T= total concentration of acrylamide and bisacrylamide: T= 48 + 1.5 = 49.5 %

C= percentage of cross-linker relative to the total concentration: C= (1.5 /(48.1.5))*100=3 %

48% acrilamide +1.5% bis-acrilamide (32:1)

Prepare in the chemical hood. Filtrate and keep at 4 ºC.

Ammonium persufate (APS) 10% (prepare just before use)

Distaining solution

7.5 % glacial acetic acid

45% methanol

Store in chemical hood. After use, recycle by filtration through activated charcoal.

Gel buffer 3x

3 M Tris-Hcl pH 8.45

0.3 % SDS

Heat a while to facilitate dissolution. Filtrate and keep at room temperature.

Staining solution (comassie blue R-250)

0.5 % comassie brilliant blue R-250 (Sigma)

7.5 % glacial acetic acid

45% methanol

Filtrate with whatman paper nº 1 and store in the dark in a chemical hood.

5x stock sample buffer (without urea)

0.5 M sucrose

15% SDS

312.5 mM Tris

10mM Na_2EDTA

Heat a while to facilitate dissolution. Adjust pH to 6.9 with concentrate HCL. Adjust volume, filtrate and keep in 0.1 ml aliquots at 4ºC.

TEMED (tetramethylenediamine) (BIO-RAD)

2x "working solution" (prepare just before)

750 µl ddH_2O

25 µl β-ME

25 µl bromophenol blue (BPB) 0,05%

500 µl 5 x stock sample buffer

Tricine SDS-PAGE – 10% polyacrylamyde gel with 0,3 % bisacrylamide

Resolving gel 10 % T, 3% C (2x5 ml)

Glycerol – 1g

AB MIX S 49,5% T; 3% C –2 Ml

Gel buffer 3x – 3,33 ml

ddH_2O – 3.87 ml

APS 10% - 50 µl

TEMED – 5 µl

Add with a 5ml syringe or a micropipette to Minigel apparatus (BIO- RAD), to cast two gels, until 1cm below the end of each comb. Add overlay of butanol-1 and let polymerize in the

chemical hood for 1 h. remove overlay and wash with bidistilled water. Dry with filter paper before apply the stacking gel.

Stacking gel 4% T, 3% C (2x2 ml)

AB MIX S 49,5% T; 3% C – 332 µl

Gel buffer 3x – 1 ml

ddH_2O – 2.67 ml

APS 10% - 30 µl

TEMED – 3 µl

Add to the top of the resolving gel, avoiding bubble formation. Let polymerize in the chemical hood for 1 h.

Samples:

5 µl membrane sample + 5 µl 2x "Working solution"

Denature for 30 min in a 40 ºC bath.

Marker:

Perform gel electrophoresis at 100 V for about 1h 30min.

1. Stain the gel for 30 min with staining solution.
2. Distain the gel by changing distaining solution.
3. Wash with bidistilled water and dry the gel between cellophane paper sheets.

II. Frequently used buffers and solutions

10X Stock Phosphate-buffered Saline (PBS)/Liter

NaCl 80 g

KCl 2 g

Na_2HPO_4 26.8 g

KH_2PO_4 2.4

pH 7.4

50X TAE Buffer/Liter

Tris base 242 g

Acetic acid 57.1 g

0.5 EDTA, pH 8.0 100ml

pH 8.5

TAE 50X

242 g/l Tris-base

57.1 ml/l Glacial acetic acid

18.612 g/l EDTA

DNA loading buffer 10X

30% (w/v) Ficoll

0.25% (w/v) Xylene Cyanol FF

0.25% (w/v) Bromophenol Blue

0.5 M EDTA, pH 8.0

30% Acrylamide-stock solution

29.2%(w/v) Acrylamide

0.8% (w/v) Bisacrylamide

APS 10% (w/v)

Laemmli loading buffer 2X

0.1M Tris-HCl, pH 6.8

2% (w/v) SDS

2% (v/v) ß-Mercaptoethanol

20% (v/v) Glycerol

0.002%(w/v) Bromophenol Blue

SDS electrophoresis buffer

0.19 M Glycine

25 mM Tris-base

0.1% (w/v) SDS

Coomassie fixing solution

25% (v/v) Isopropanol

10% (v/v) Glacial acetic acid

Coomassie staining solution

10% (v/v) Glacial acetic acid

60 mg/l Coomassie brilliant blue R250

TE

10 mM Tris-HCl, pH 7.4-8.0

1 mM EDTA, pH 8.

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